Positive and negative regulation of T cell activation via CD80

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This thesis is dedicated to
my wife Alexandra and my recently born son Orestis

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CONTENTS

Summary

The events that influence T cell activation have been under considerable investigation during the last decade. The discovery of CD28 and other costimulatory molecules that are thought to enhance T cell responses have been a central point in this research activity, but CD28 has been suggested to be the most important costimulator mainly due to its ability top prevent anergy of T cells. Interest has been further heightened since the discovery of the CD28 homologue, CTLA-4, which is suggested to negatively regulate T cell activation instead. In an attempt to explore the function of these two receptors this study has investigated the role of CD80, one of the two natural ligands for both CD28 and CTLA-4, in T cell activation. The role of CD80 as a costimulator and its importance in the induction of proliferation, IL-2 and specific transcription factors that regulate IL-2 gene expression, was examined. In contrast to many previous studies, the data presented here suggest that CD28 costimulation is not always accompanied by IL-2 production and that the latter is not necessary for the proliferative effect of CD28, suggesting a "rethink" on the pre-eminence of IL-2 as a T cell proliferative cytokine. The studies presented here also demonstrate the ability of CD80 to negatively regulate T cell activation. Specifically, the data show that whereas CD80 is a good costimulator of PMA activated T cells, the additional presence of a high strength calcium signal can actually reverse this costimulatory function to a negative regulatory one. This effect is specifically mediated by the ligation of CD80 to CTLA-4, but the data also suggest that CD28 may also be required at the same time. Overall the data suggest that signals from both the TCR and the CD28 receptor may ultimately determine whether CD80 will continue to positively regulate activation by ligating CD28 or negatively control T cells by ligating CTLA-4.

Abbreviations

Ab	Antibody
Ag	Antigen
AICD	Activation Induced Cell Death
AP50	Adapter Protein 50
AP1	Activator Protein 1
APC	Antigen Presenting Cell
APS	Ammonium Persulphate
aSMase	Acidic Sphingomyelinase
ATCC	American Type Culture Collection
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
CD	Cluster of Differentiation
cDNA	Complementary Deoxyribonucleic Acid
CHO	Chinese Hamster Ovary cells
CO2	Carbon Dioxide
CTL	Cytotoxic T lymphocyte
CTLL	Cytotoxic T cell Leukaemic Line
CTLA	Cytotoxic T Lymphocyte Antigen
CREB	cAMP Responsive Element Binding protein
CsA	Cyclosporin A
DAG	Diacylglycerol
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleoside triphosphates
DTT	Dithiothreitol
EAE	Experimental Allergic Encephalomyelitis
EDTA	Ethylenediaminetetracetic acid
ELISA	Enzyme Linked Immunosorbant Assay
EMSA	Electromobility Gel Shift Assay
ERK	Extracellular signal Receptor Kinase
FACS	Fluorescence Activated Cell Sorter
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
FL	Fluorescence
FSC	Forward light Scatter
GAP	GTPase Activating Protein
GEF	Guanine nucleotide Exchange Factor
GM-CSF	Growth Maturation Colony Stimulating Factor

GSK-3	Glycogen Synthase Kinase 3
GTP	Guanosine Triphosphate
grb2	Growth factor Receptor Bound protein 2
^{3}H-Thymidine	Tritiated Thymidine
HLA	Human Leukocyte Antigen
IDDM	Insulin Dependent Diabetes Mellitus
IFN	Interferon
Ig	Immunoglobulin
IkB	Inhibitor of kB
IL	Interleukin
IL-2Rα	Interleukin 2 Receptor, α chain
IP_3	Inositol 1,4,5 trisphosphate
ITAM	Immunoreceptor Tyrosine based Activation Motif
ITK	Inducible T cell Kinase
JNK	Janus (or c-Jun N terminal) Kinase
MAPK	Mitogen Activating Protein Kinase
MAPKK (or MEK)	Mitogen Activating Protein Kinase Kinase
MAPKK (or MEKK)	Mitogen Activating Protein Kinase Kinase Kinase
MHC	Major Histocompatibility Complex
mRNA	Messenger Ribonucleic Acid
NK	Natural Killer
NFAT	Nuclear Factor of Activated T cells
NF-kB	Nuclear Factor kappa B
NGF	Nerve Growth Factor
NGFR	Nerve Growth Factor Receptor
PAK	p21 Activated Kinases
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffer Saline
PCD	Programmed Cell Death
PDGF	Peptide Derived Growth Factor
PDGFR	Peptide Derived Growth Factor Receptor
PHA	Phytohaemagglutinin
P/I	PMA and Ionomycin
PI3K	Phosphatidylinositol-3 kinase
PIP_2	Phosphatidylinositol 4,5 bisphosphate
PIP_3	Phosphatidylinositol 3,4,5 trisphosphate
PKB	Protein Kinase B
PKC	Protein Kinase C
PLC	Phospholipase C
PMA	Phorbol Myristate Acetate

PMSF	Phenylmethylsulfonyl fluoride
PNK	Polynucleotide Kinase
PP	Protein Phosphatase
PTK	Protein Tyrosine Kinase
PTP	Protein Tyrosine Phosphatase
RC	Response Complex
RE	Response Element
RNA	Ribonucleic Acid
RT	Reverse Transcription
SAPK	Stress Activated Protein Kinase
SDS	Sodium Dodecyl Sulphate
SHP	SH2 containing Protein Tyrosine Phosphatase
SLAM	Signalling Lymphocytic Activation Molecule
SMase	Sphingomyelinase
SSC	Saturated Sodium Chloride
STAT	Signal Transducers and Activators of Transcription
TAE	Tris Acetate with EDTA
TBE	Tris Borate with EDTA
TCR	T Cell Receptor
TEMED	NNN'N'-tetramethylethylenediamine
Th	T helper
TNF	Tumour Necrosis Factor
TOR	Target of Rapamycin
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

1.1: T CELLS AND THE IMMUNE RESPONSE.

Several different cell types are involved in the immune system and a complex set of interactions between these cells, together with their correct activation is responsible for the efficient action of the immune system against disease. Thymus derived lymphoid cells (T cells) play a key role in these processes, since they are responsible for recognising foreign antigens and initiating further events, including their own activation and propagation (Clark and Ledbetter, 1994). Prior to this, cells known as antigen presenting cells (APCs) which include monocytes, macrophages, dendritic cells and B cells, uptake the foreign antigen which they process and present on their surface bound to major histocompatibility complex (MHC) molecules (Harding, 1994). It is this complex that is recognised by the T cell receptor (TCR) and induces the necessary signals that will mediate T cell activation.

T cell activation is characterised by cell enlargement, proliferation and expansion and the production of cytokines that mediate specific effector functions for the cells. However, for this to happen, the presentation of an antigen is not enough and a second signal, initiated after the engagement of CD28 receptor is generally required (Bretscher et al 1970; Linsley et al., 1991a; Azuma et al., 1992; Gimmi et al., 1993; Tan et al., 1993; Mueller et al., 1989; June et al., 1994). CD28 is present on the surface of naive / resting T cells and interacts with molecules that belong to the B7 receptor family, present on the APCs (Weaver and Unanue, 1990; June et al., 1994; Liu and Janeway, 1992). In the absence of this CD28 engagement, TCR signals can not activate the cells which instead become anergic (Jenkins et al., 1990; Tan et al., 1993; Gimmi et al., 1993; Yi-quan et al., 1997) or they even die via apoptosis (Groux et al., 1993; Liu and Janeway Jr., 1990; Russell, 1995).

1.1.1: Recognition of MHC molecules: The T cell receptor.

MHC molecules are divided in to two types, differing in their structure and the type of peptide they present on the surface as antigen. Specifically, MHC class I molecules present peptides that have derived from cytosolic antigens (e.g. viral proteins synthesised in infected cells) (Babbit et al., 1985; Townsend et al., 1989). In contrast MHC class II molecules present antigens that have been internalised in APCs via endocytosis (e.g. from engulfed bacteria by macrophages) (Lotteau et al., 1990; Neefjes et al., 1990). Both class I and class II MHC molecules are recognised by the TCR, although different co-receptors determine the specificity of the interactions (Viola et al., 1997b; Eckels et al., 1988; Teh et al., 1988). Thus, antigenic peptides bound to MHC class I molecules are recognised by $CD8^{+ve}$ cytotoxic T cells which then mediate a response, during which the APC lyses after the destruction of its ionic and osmotic balance, or dies via apoptosis (Harding and Allison, 1993; Sigal et al., 1998; Berke, 1995). In contrast antigens presented in MHC class II molecules are recognised by $CD4^{+ve}$ T helper cells (Clark and Ledbetter, 1994). In this case a response is initiated during which cytokines are released to stimulate T and B cell growth and maturation.

The T cell receptor (TCR), a glycoprotein belonging to the immunoglobulin superfamily, is vital for the recognition of foreign antigens. Overall, it is a disulphide linked heterodimer, made up of one α (55kDa) and one β (45kDa) chain, each with an outer domain which contains clonally variable regions important for the specific recognition of the antigen presented by the MHC molecule (Blackman et al., 1990). Most T cells contain TCR$\alpha\beta$ molecules on the surface. Some T cells however contain one γ and one δ chain instead and make up a distinct set of T cells with less clear functions (Arstila, 1996). Close to the TCR and associating non-covalently is the CD3 complex, which contains three chains, γ (25kDa), δ (20kDa) and ε (20kDa), arranged in pairs (Wegener et al., 1992). Also non covalently associated with TCR and CD3 are $\zeta\zeta$ and $\zeta\eta$ chains forming the complete TCR/CD3

complex. The γ, δ, ε, ζ and η chains are involved in the assembly of the complex and the expression of TCRα/β on the surface and MHC recognition, but are also thought to perform signalling functions for the TCR (Rudd et al., 1994; Wegener et al., 1992).

1.1.2: The two signal model of T cell activation: Role of CD28 and CTLA-4.

1.1.2.1: The costimulatory potential of CD28.

1.1.2.1a: The CD28 receptor.

CD28 is expressed on 95% of $CD4^{+ve}$ and 70-80% of $CD8^{+ve}$ resting T cells. It is also present on activated T cells, plasma cells, certain natural killer (NK) cells and thymocytes (Aruffo and Sedd, 1987; Gross et al., 1990; Sfikakis et al., 1995; Gross et al., 1992). On the cell surface, CD28 is expressed as a disulphide linked homodimer (Gross et al., 1992; June et al., 1994). Each subunit is a 44kDa glycoprotein consisting a single V_H-like domain in the extracellular region, a transmembrane region and a short (41 amino acids) cytoplasmic tail which is the highest conserved region and includes a PI3K binding motif (Truitt et al., 1994; Prasad et al., 1994; Gross et al., 1990).

The levels of CD28 on the T cells decrease after engagement both in terms of mRNA and cell surface expression (Linsley et al., 1993; Cefai et al., 1998), but quickly return to normal levels and are even upregulated once activation is established, a process that probably enhances costimulation. Eventually CD28 mRNA levels and subsequently the amount of CD28 on the surface falls to basal levels. Interestingly in the absence of CD28 engagement, activation with CD3 or PMA increases the levels of CD28 (Gross et al., 1992). This is possibly due to the fact that the CD28 gene has an AP1 site in its promoter, but increased activation may also take place at the post-transcriptional level.

1.1.2.1b: Role of the CD28 receptor on T cell activation and T cell survival.

The ability of CD28 to aid T cell activation and the production of cytokines such as IL-2 is well established (Linsley et al., 1991a; Tan et al., 1993; Harding et al., 1992; Galvin et al., 1992). This requirement of CD28 for productive T cell activation is clearly suggested by CD28 deficient mice whose T cells respond poorly to lectins and produce low levels of IL-2 (Shahinian et al., 1993). More strikingly, B cell activation and germinal centre formation (both T cell dependent processes) are highly defective (Ferguson et al., 1996; Chen et al., 1992; Shahinian et al., 1993). Interestingly, CTL responses are not affected suggesting that alternative costimulators may play a role. Further studies have shown the importance of costimulation by CD28 using soluble antagonists of CD28 or CD28 ligands, which block CD28 engagement. One such soluble antagonist is CTLA-4Ig which consists of the extracellular region of CTLA-4 (CD152), a CD28 homologue that can bind the same ligands as CD28 but with higher affinity, fused with the constant region of human Ig-G1 (Linsley et al., 1992b). CTLA-4Ig has been very useful in the studies of CD28, because by binding its ligands blocks costimulation and has proved the importance of CD28 by generating tolerance against transplants in mice (Lenschow et al., 1992) and rats (Lin et al., 1993; Turka et al., 1992). Thus, by administrating CTLA-4Ig with a transplant, rejection is prolonged or even avoided completely. The costimulation process is also seen in transgenic mice with a CD28 ligand in pancreatic β cells. Only when DR4 and an antigen (lymphocyte choriomeningitis virus glycoprotein) are co-transfected, are T cells activated and attack the β-pancreatic cells resulting in diabetes (Harlan et al., 1994). However, probably the most direct *in vivo* evidence for costimulation, came from tumour studies (Chen et al., 1992; Baskar et al., 1993). A CD28 ligand and a tumour antigen (e.g. E7 protein from human papilloma virus-HPV16) have to be co-transfected in tumour cells to be rejected, whereas either of them transfected alone has no or low effect, unless the tumour cell already expresses the missing stimulus.

CD28 is therefore important for the activation of T cells and is thought to determine whether T cells selected by TCR engagement will be expanded (Wells et al., 1997) and at the same time increase their overall activation (Thompson et al., 1993). It has also been suggested that CD28 is able to reduce the threshold number of TCRs required for T cell activation (Viola and Lanzavecchia, 1996) and therefore increase antigen sensitivity. In any case however, CD28 does not simply reinforce TCR signals since high strength TCR stimulation can not lead to full T cell activation (Wells et al., 1997; Damle et al., 1993; Mueller et al., 1990). Instead CD28 is also thought to initiate unique pathways that aid the productive activation of the T cells in terms of proliferation and cytokine production (Gimmi et al., 1991; Reiser et al., 1992; June et al., 1987). IL-2 is thought to be important for S phase progression (Shibuya et al., 1992; Taniguchi and Minami, 1993; Miyazaki et al., 1995) and has been suggested to mediate the costimulatory activity of CD28 (Kuiper et al., 1994; Jenkins, 1992; Nunes et al., 1993). In this respect, T cells treated with anti-CD3 and anti-CD28 antibodies in the presence of anti-IL-2Rα and anti-IL-2 blocking antibodies become anergic (Jenkins, 1992). However the ability of CD28 to increase proliferation of T cells is not always accompanied by IL-2 (Boussiotis et al., 1993; Edmead et al., 1996; Khoruts et al., 1998) and not all CD28 costimulatory signals can be substituted by IL-2 (Sperling et al., 1996; Levine et al., 1997; Schweitzer and Sharpe, 1998). Furthermore, studies with $CD4^{+ve}$ cells from $IL\text{-}2^{-/-}$ mice have shown that CD3 and CD28 costimulation can take place although less efficiently (Razi-Wolf et al., 1996), whereas other studies have shown that $CD4^{+ve}$ cells of $IL\text{-}2^{-/-}$ mice responded normally to antigen in *vivo* (Khoruts et al., 1998).

The above observations suggest that IL-2 does not necessarily mediate the costimulatory action of CD28 and that other targets may be equally important. In this respect CD28 may be inducing signals that promote the survival of the cells. In fact, studies in our laboratory have clearly shown the ability of correctly activated T cells to escape cell death after TCR activation with antigen (McLeod et al., 1998). Other studies also support the concept that CD28 aids survival in the thymus

(Wagner et al., 1996; Shi et al., 1995) and the periphery (Noel et al., 1996; Sperling et al., 1996; Daniel et al., 1997), by generally showing that T cells activated via the CD28 receptor survive longer in culture (Lucas et al., 1995; Levine et al., 1997). Although not universally accepted (Vella et al., 1997; Boussiotis et al., 1997), T cell survival may be one of the main roles of CD28. Thus, CD28 may participate in the increase of the general metabolic rate of the T cells and promote a more effective and sustained proliferation. CD28 may even not be required for the primary T cell activation / division, but required for maintenance. This would explain the transient proliferative responses of $CD28^{-/-}$ mouse T cells (Sperling et al., 1996; Shahinian et al., 1993; Lucas et al., 1995). The anti-apoptotic effects of CD28 are thought to be mediated by the activation of PKB and subsequent phosphorylation of BAD (Boise et al., 1995b; Noel et al., 1996; Sperling et al., 1996; Levine et al., 1997). When in the phosphorylated state, BAD stops interacting with bcl-X_L which is let free to perform its cell survival role (Boise et al., 1993b; Boise and Thompson, 1997; Chao et al., 1995). Generally, bcl-X_L requires a threshold to act and TCR stimulation is thought to only induce low and insufficient levels unless CD28 is also engaged (Boise et al., 1995b; Sperling et al., 1996).

1.1.2.2: CTLA-4 (CD152), a receptor that antagonises activation.

CTLA-4, belongs to the same family of receptors as CD28 (Brunet et al., 1987; Harper et al., 1991) and shares the same ligands (Linsley et al., 1991b; Azuma et al., 1993a). Unlike CD28 however, it is not expressed on resting T cells, but is upregulated after TCR and / or CD28 engagement (Finn et al., 1997; Freeman et al., 1992; Linsley et al., 1992a; Lindsten et al., 1993). CTLA-4 expression is localised in the cytoplasm and is suggested to appear transiently at the surface during the initial stages of activation (Linsley et al., 1996; Alegre et al., 1996; Leung et al., 1995). Specifically, immediately after activation CTLA-4 accumulates in certain perforin containing secretory vesicles (Linsley et al., 1996). These are thought to be directionally targeted towards the cell-cell contact site (i.e. TCR / MHC interactions) and therefore exocytose CTLA-4 at the surface. A calcium dependent signal is

thought to aid this directional exocytosis (Linsley et al., 1996). Once at the surface, endocytosis takes over by the formation of clathrin coated pits. CTLA-4 interacts with AP50 which is part of these vesicles, via its Y_{210}VKM motif when the receptor is unphosphorylated at the tyrosine (Y) residue (Zhang and Allison, 1997; Bradshaw et al., 1997; Chuang et al., 1997; Shiratori et al., 1997). Thus, CTLA-4 phosphorylation at Y_{210} after activation (Schneider et al., 1995; Bradshaw et al., 1997) may be required to prevent endocytosis, keep CTLA-4 on the surface and permit its functions. However, as studies in mice have shown, stable surface expression is not seen until 2-3 days after activation (Alegre et al., 1996; Lindsten et al., 1993; Linsley et al., 1992a) and even then at very low levels compare to that of CD28. Despite this low and transient CTLA-4 surface expression, its function may be compensated by the fact that it binds its ligands with 20-100 times higher affinity than CD28 (Linsley et al., 1994; Greenfield et al., 1997). Additionally, the ability of CTLA-4 to signal intracellularly at the initial stages of T cell activation can not be ruled out.

The function of CTLA-4 has been of considerable debate since its discovery, with some reports using antibodies for the receptor initially suggesting a positive costimulatory and / or a negative role (Linsley et al., 1992a; Chen et al., 1992; Walunas et al., 1996a; Krummel and Allison, 1995; Krummel and Allison, 1996; Walunas et al., 1994; Blair et al., 1998), while others characterising it as an agent of death (Gribben et al., 1995; Scheipers and Reiser, 1998). However, cross-linked CTLA-4 antibodies prevent activation by CD3 and CD28 (Krummel and Allison, 1996; Walunas et al., 1996a; Krummel and Allison, 1995) suggesting that CTLA-4 opposes T cell activation and the additive effect of CTLA-4 soluble antibodies observed in some cases may simply have been the result of blocking the receptor. Furthermore, blocking B7 responses via CTLA-4Ig or anti-B7 antibodies increases T cell activation by anti-CD3 and anti-CD28 antibodies. CTLA-4 blockade has also been shown to increase anti-tumour immunity, clearly showing its negative function (Chen et al., 1992; Leach et al., 1996). However, the most convincing in *vivo*

evidence came from CTLA-4$^{-/-}$ knockout mice, which were found to suffer from a severe lymphoproliferative disorder, leading to death after 3-4 weeks of age due to myocardial failure after the uncontrolled infiltration of active T cells (Waterhouse et al., 1995; Chambers et al., 1997). The ability of CTLA-4Ig to prevent the lymphoproliferative disorder on the CTLA-4$^{-/-}$ mice (Tivol et al., 1997) suggests that CD28 induced signals are left uncontrolled in these mice. However, CTLA-4 has been shown to decrease proliferation and IL-2 production (Fallarino et al., 1998; Krummel and Allison, 1996) and prevent allograft rejection (Lin et al., 1998) in CD28$^{-/-}$ mice as well. It is therefore possible that CTLA-4 prevents the general activation of the cells and not specifically CD28 mediated signals.

Overall, a balance between CD28, CTLA-4 and TCR signals must determine the final outcome of T cell stimulation by possibly regulating initiation of responses. Although CTLA-4 is not present on resting T cells, it is induced very quickly as studies have shown recently (Linsley et al., 1996; Finn et al., 1997; Freeman et al., 1992). By acting early, CTLA-4 is suggested to prevent G1 to S transition and not to affect death (Walunas et al., 1996a; Krummel and Allison, 1996). Additionally, CTLA-4 may act at later stages and stop ongoing processes. As a result CTLA-4 may prevent activation induced cell death and at the same time allow memory cells to be generated (Chambers et al., 1996). Although such an important role of CTLA-4 is suggested by the fact that the expression of CTLA-4 is high and stable only 2-3 days after activation (Finn et al., 1997; Freeman et al., 1992; Linsley et al., 1992a; Lindsten et al., 1993), the ability of the CD28 / CTLA-4 ligands to further proliferate activated T cells (Edmead et al., 1996) contrasts with this idea. It must be noted however that all these suggestions have been based on studies that utilised antibodies for both the CD28 and the CTLA-4 receptor which ignore the competition of the two receptors for their common ligand. Thus, further studies with the natural ligands are important for the understanding of the regulatory functions of both CD28 and CTLA-4.

1.1.2.3: The CD28 / CTLA-4 ligands: The B7 receptor family

As mentioned above both CD28 and CTLA-4 interact with the same ligands that belong to the B7 family of receptors. Two members, present on APCs and belonging to the immunoglobulin superfamily, are well characterised. They share about 26% amino acid identity and bind their receptors at a MYPPY motif present in both CD28 and CTLA-4. Initially known as B7-1 (Linsley et al., 1991a; Freeman et al., 1989; Freeman et al., 1991) and B7-2/B70 (Azuma et al., 1993a; Freeman et al., 1993b; Freeman et al., 1993a), they are now referred to as CD80 and CD86 respectively. The ability of the CD80 antibody BB1, to bind CD80 (but not CD86), but stain certain cells that do not express CD80 suggested initially the presence of an alternative third B7 molecule (Murakami et al., 1996; Boussiotis et al., 1993). However, recent evidence suggested that this is due to the ability of BB1 to bind CD74 (class II invariant chain) (Freeman et al., 1998).

Both CD80 and CD86 are glycosylated proteins encoded by genes close together in the genome. They bind CD28 and CTLA-4 with similar affinity although as mentioned above CTLA-4 binds both ligands stronger, possibly due to the IgC domain present in the B7 molecules (Linsley et al., 1994). With a few exceptions the induction of CD80 and CD86 occurs using similar stimuli, including costimulation itself, possibly because of their close proximity in the genome (Hatchcock et al., 1994; Nabavi et al., 1992). However, their expression pattern differs. Generally CD80 is expressed at low levels on resting APCs but is increased after activation (Freeman et al., 1991; Freedman et al., 1991; Hart et al., 1993). On the same cells CD86 is constitutively expressed, but again its level increases after activation (Hatchcock et al., 1994; Fleischer et al., 1996). Resting T cells do not express any costimulatory molecules but their repeated activation results in CD86 firstly, followed by CD80 expression (Sansom and Hall, 1993; Azuma et al., 1993b; Wyss-Coray et al., 1993). Although this suggests that T cells may actually act as APCs, the activation of T cell clones by T cells as APCs leads to anergy whereas in resting T cells, antigen presentation by T cells leads to CTL activity (Pichler and Wyss-

Coray, 1994; Chai et al., 1998). Thus, antigen presentation by T cells may negatively regulate T cell activation. In that respect CD86 on T cells has been suggested to preferentially bind CTLA-4 (Greenfield et al., 1997).

The fact that CD86 is constitutively present on APCs suggests that it may be the primary costimulatory ligand for CD28 that initiates an immune response and the costimulation of unprimed lymph nodes. In fact *in vivo* proliferation of T cells is blocked more by anti-CD86 than by anti-CD80 (Razi-Wolf et al., 1996; VanParijs et al., 1997). Similarly CD86 deficient mice show a worse phenotype than CD80 deficient mice although neither of them show abrogated responses and many functions seem to be overlapping (Borriello et al., 1997). In contrast to CD86, CD80 is upregulated after activation, suggesting that its main purpose is to maintain an immune response (Bluestone, 1995; Thompson, 1995). It is also suggested however that CD80 may be the primary ligand for CTLA-4 a concept supported by the fact that CD86 dissociates faster that CD80 after binding CTLA-4 (Linsley et al., 1994).

Overall CD28/CTLA-4 on T cells and CD80/CD86 molecules on the APCs can interact and regulate T cell activation and TCR signalling by either promoting a "GO" signal or a "STOP" signal on the initiation of T cell activation (**Figure 1.1**). Clearly, the competition between CD28 and CTLA-4 for their common ligands may clearly be an important factor that determines the dominance of one outcome over another and will clearly depend on the expression levels of CD80/CD86 and CD28/CTLA-4. Because the studies performed so far have mainly concentrated on the effects of antibodies for these two receptors, this competition has not been taken in to account and is lacking in the literature.

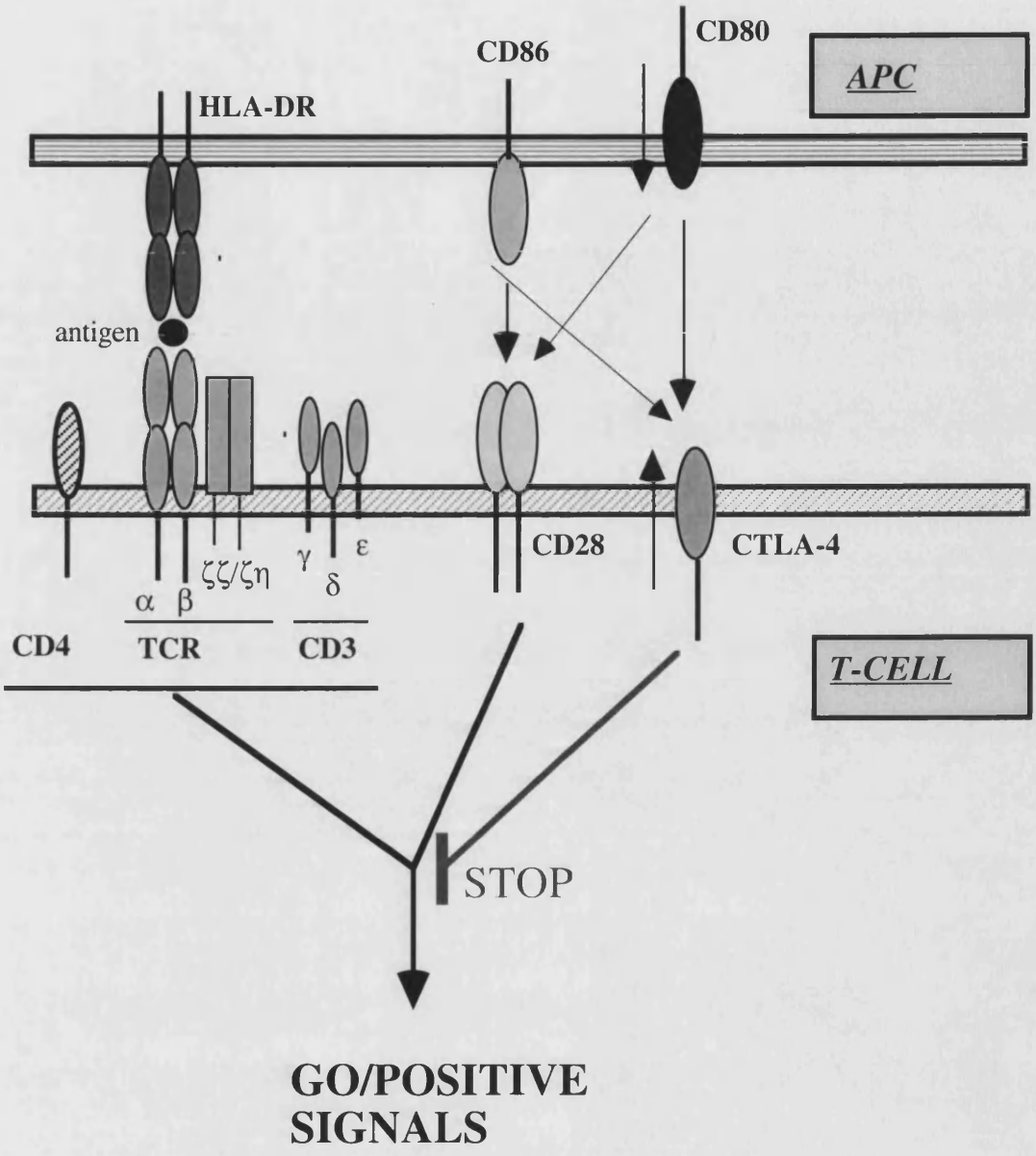

Figure 1.1: T cell receptors and APC counter-receptors that regulate T cell activation. Engagement of the T cell receptor (TCR) takes place by an antigen presented by HLA-DR molecules on the surface of the antigen presenting cell (APC). Simultaneous engagement of the CD28 receptor induces the necessary signals that synergise with TCR and activate T cells. The CD28 homologue CTLA-4, interacts with the same ligands as CD28 but is thought to oppose these activation signals.

1.1.3: T cell activation in the absence of CD28.

1.1.3.1: Anergy

Several studies have shown that in many cases, cells stimulated by the TCR alone fall in a state of anergy and the ability of CD28 to rescue T cells from this state is well documented (Harding et al., 1992; Gimmi et al., 1993; Galvin et al., 1992; Mueller et al., 1989; Tan et al., 1993). Overall the cells become tolerant and are characterised by the lack of proliferation and any cytokine production such as IL-2 (DeSilva et al., 1991; Gilbert and Wiegle, 1993; Mueller and Jenkins, 1995; Jenkins et al., 1990; Ramsdell and Fowlkes, 1992; Davis et al., 1989). Although the cells are unable to respond after TCR and CD28 engagement (Harding et al., 1992; Gimmi et al., 1993; Galvin et al., 1992) they are not dead and exogenous IL-2, but not IL-4 can rescue the cells from this state and increase proliferation, although death follows shortly after IL-2 deprivation (Gimmi et al., 1993; Yi-quan et al., 1997; Mueller et al., 1991; Beverly et al., 1992).

The lack of IL-2 production during anergy was initially thought to be a passive result after TCR engagement due to the lack of certain signals (Fields et al., 1996; Carmella et al., 1996; Mueller and Jenkins, 1995). However TCR can signal alone (Damle et al., 1993; Shahinian et al., 1993; Lucas et al., 1995) and lead to CD69 induction, IL-2Rα chain expression and LFA-1 increase. Additionally, certain cytokines may be induced, depending on the cell type utilised, although this has not been seen with "real" T cells (Harding et al., 1992; Yi-quan et al., 1997). In addition, cyclohexamide (a protein synthesis inhibitor) can prevent anergy suggesting that active signals govern anergy (Jenkins, 1992; Gilbert and Wiegle, 1993). Further supporting this, certain negative regulatory elements are thought to be present at the IL-2 promoter which are mainly occupied in anergic T cells and may negatively regulate IL-2 gene transcription (Williams et al., 1991; Becker et al., 1995; Kitagawa-Sakakida and Schwartz, 1996). More significantly an inhibitor of $p21^{Ras}$ (an important T cell activation molecule) called Rap1 may play a role in

anergy, since its levels are high in anergic cells but are decreased by CD28 (Reedquist and Bos, 1998), the costimulatory molecule that prevents anergy.

1.1.3.2: Apoptosis

An alternative route that may follow TCR engagement in the absence of costimulation, is apoptosis or programmed cell death (PCD) (Liu and Janeway Jr., 1990; Groux et al., 1993). Generally, T cells die by either active death (antigen driven activation induced cell death-AICD) or passive death (Cohen et al., 1992). The latter may result after cytokine withdrawal (Lenardo, 1996). AICD on the other hand acts to limit the effect of persistent antigen. Usually it does not take place after primary infection but the cells become sensitive to AICD during the second antigen challenge (Lenardo, 1996; Russell et al., 1991; Salmon et al., 1994). Entry in the S phase of the cell cycle takes place prior to AICD (Boehme and Lenardo, 1993) and in T cells specific signals mediated by the Fas (CD95) receptor are required (Itoh et al., 1991; Nagata and Suda, 1995; Russell et al., 1993). Fas (CD95) belongs to the TNF family of receptors (Smith et al., 1994), is upregulated by the TCR and is thought to act as a death agent and kill cells that have performed their function (Nagata, 1997; Watanabe-Fukunaga et al., 1992; Russell, 1995). It is also suggested that Fas may mediate peripheral clonal deletion of T cells that recognise self antigens (Nagata, 1997) or that recognise antigens unproductively (McLeod et al., 1998). Finally the immune privilege sites which can not tolerate inflammation and dangerous immune responses (e.g. testis, eye) may utilise the Fas-ligand expressed on cells at the vicinity to kill activated T cells that arrive and express Fas (Nagata, 1997).

1.1.3.3: CD28 independent costimulation.

The fact that not all T cells express CD28 (Gross et al., 1992) together with the ability of CD28 deficient mice to mount certain immune responses (Shahinian et al., 1993; Ferguson et al., 1996), suggested that the importance of CD28 is not universal. High antigen doses can actually lead to a short-lived but nevertheless

vigorous response (Damle et al., 1993; Chen et al., 1992, Lucas et al., 1995). In other cases alternative costimulatory molecules support TCR stimulation, although in many cases the effect is indirect due to an increased adhesion between T cells or T cells and APCs (Springer, 1990; Yashiro et al., 1998). Importantly, compared to other costimulatory molecules, CD28 is able to act *in trans* with the primary signal, clearly showing its important signalling ability (Sansom et al., 1993). In most cases additional costimulators are thought to act together with CD28 and potentiate or control costimulation. Examples include CD2 (Kato et al., 1992; Parra et al., 1997), CD11 (DeBenedette et al., 1997), CD43 (Sperling et al., 1995), CD44 (Naulokas et al., 1993), 4-1BB (Melero et al., 1998; Kim et al., 1998b) and CD30 (Gilfillan et al., 1998). In other cases however some molecules have been seen to act alone on activated T cells (e.g. SLAM (Aversa et al., 1997; Cocks et al., 1995) and CD30 (Gilfillan et al., 1998)). Furthermore, 4-1BB (DeBenedette et al., 1997) and CD6 (Osorio et al., 1998) have been seen to act in the absence of CD28. Overall it is not clear which molecules are essential for which type of responses, but it is likely that more than one of them control T cell activation at each time point.

1.1.4: The two signal model of T cell activation: Costimulation and the control of the immune system.

1.1.4.1: Costimulation in the thymus.

T cells derive from the bone marrow, but develop in the thymus, were they undergo two types of selection. During negative selection, about 95% of thymocytes die via apoptosis because they are potentially autoreactive or because they do not rearrange their TCR$\alpha\beta$ (or $\gamma\delta$) genes correctly (Sha et al., 1988; Nikolic-Zugic, 1991; Rocha and von Boehmer, 1991; Blackman et al., 1990; Ashtonrickardt et al., 1994). Most of the cells that survive end up with TCR$\alpha\beta$ on their surface and either CD4 or CD8. Negative selection can still continue in these matured single positive thymocytes if the TCR interacts strongly with self MHC molecules (clonal deletion). However positive selection also takes place and selects T cells that recognise foreign

peptides presented by self MHC molecules (Sha et al., 1988; Schwartz, 1989). Overall the thymus is an education centre for the T cells where they learn to recognise self and non-self antigens (Kronenberg, 1991; Schwartz, 1989; Morahan et al., 1989).

$CD28^{-/-}$ and $CTLA\text{-}4^{-/-}$ mice show normal positive and negative selection (Chambers et al., 1997; Walunas et al., 1996b). Although this clearly shows that costimulation is not vital during selection, certain studies suggest that CD28 may perform subtle changes / pressures in thymic selection by increasing the rate of expansion and survival of $CD28^{+ve}$ cells compare to $CD28^{-ve}$ cells. Specifically it has been found that from a mixture of $CD28^{+ve}$ and $CD28^{-ve}$ thymocytes a larger percentage of $CD28^{+ve}$ cells than $CD28^{-ve}$ cells make it to the periphery (Walunas et al., 1996b). Also supporting a role for CD28, its expression levels are highest during negative selection (Gross et al., 1992). Additionally, double positive thymocytes can be rescued of glucocorticoid or CD3 induced death if CD28 ligands or CD28 antibodies are also given to the cells (Wagner et al., 1996; Shi et al., 1995). Interestingly the same studies also suggested that antibodies for the CTLA-4 receptor can also rescue thymocytes from glucocorticoid induced death (Wagner et al., 1996). It is possible however that in this case CTLA-4 signalling prevents T cell stimulation as mentioned above and as a result prevents activation that is ultimately required for apoptosis to follow.

1.1.4.2: Costimulation and autoimmunity.

Once T cells are released to the circulation, extra safeguards against self reactivity also exist and comprise the mechanisms that aid immunologic tolerance (Rocha and von Boehmer, 1991; Kronenberg, 1991; Schwartz, 1989). Anergy may be an important mechanism that aids self tolerance to APCs that have not derived from the bone marrow and therefore have no costimulatory molecules on their surface (Mueller and Jenkins, 1995). The ability of CTLA-4 to negatively regulate T cell activation is also suggested to perform important functions that mediate peripheral

tolerance (Bluestone, 1997). All these mechanisms are important and their collapse may lead to undesirable effects such as the activation of self reactive T cells and the onset of autoimmunity, including rheumatoid arthritis (RA), insulin dependent diabetes mellitus (IDDM), and multiple sclerosis (MS) which affect some 5% of the population (Kronenberg, 1991; Harlan et al., 1995). Abnormal utilisation of the costimulatory signals may be partly responsible for the generation and / or maintenance of the autoimmune character by allowing T cell activation to take place at times it would not normally happen. The constitutive expression of CD80 on dendritic cells of patients with rheumatoid arthritis, hints for a role of costimulation in autoimmunity. In fact several autoimmune diseases can be prevented by blocking costimulation via CTLA-4Ig or via the use of blocking antibodies against the CD28 receptor or its counter-receptor CD80 (Finck et al., 1994; Kuchroo et al., 1995; Harold et al., 1997). More directly, another study showed that mice with pancreatic β cells transgenic for CD80 and for lymphocytic choriomeningitis virus glycoprotein (together with MHC-DR4), but not each alone, are characterised by activated T cells and the development of autoimmune diabetes (Harlan et al., 1994). In contrast blockade of CTLA-4 has been shown to enhance experimental allergic encephalomyelitis (EAE) disease progression and establishment suggesting that the CTLA-4 pathway is responsible for downregulating autoimmunity as well (Karandikar et al., 1996).

1.1.4.3: Costimulation and peripheral tolerance

In some cases tolerance mechanisms have been found to take place even after the presentation of a non-self antigen. Theoretically any defect in the costimulatory process is capable of resulting in T cell inability to react. As also mentioned above (section 1.1.2.1b) this has been clearly seen in transplantation studies, during which the blockade of CD28 ligands via CTLA-4Ig, allowed prolonged survival and acceptance of the transplants (Lin et al., 1993; Lenschow et al., 1992). Similarly, tolerance of the immune system against cancerous cells in our body is often due to the lack of CD28 / CTLA-4 ligands on the surface of the tumour cells. However,

transfection of CD80 on the tumour cells has been found to aid the rejection of immunogenic tumours, whereas non-immunogenic tumour cells also required transfection of an antigen (Chen et al., 1992; Baskar et al., 1993). Furthermore, more recent studies have shown that blockade of the CTLA-4 receptor with antibodies can further accelerate tumour rejection (Leach et al., 1996). Thus, CTLA-4 is again shown to downregulate T cell activation and may therefore play an important role in the control of peripheral T cell tolerance. Clearly, although helpful after transplantation, tolerance is not always desirable and the understanding of the action of CD28 and / or CTLA-4 is important in our ability to control T cell activation and therefore tolerance.

1.1.4.4: Costimulation and T cell differentiation.

1.1.4.4a: General principles of T cell differentiation.

T cells are present in the periphery as precursor T cells (Thp) and after antigen presentation and costimulation they develop (with a possible intermediate Th0 stage) in to two possible subtypes (Th1 and / or Th2) that differ in the type of cytokines that they secrete and as a consequence mediate different effector functions and initiate different immune responses (Lichtman and Abbas, 1997; Thompson, 1995) **(Figure 1.2)**. It is unclear if a discreet or the whole population of activated T cells differentiates towards a specific Th phenotype but it appears that the final population is not absolutely of one type but tends to favour one phenotype over the other (Kelso, 1995; Seder and Paul, 1994). However, the specific generation of Th1 and / or Th2 cells is relevant and important for diseases since domination of one of the two phenotypes can either enhance or block disease progression and establishment (Liblau et al., 1996).

As seen in **figure 1.2**, cytokines play an important role in this process and since APCs secrete cytokines, the type of APC present at the time will also be important (Romagnani, 1992; Yoshimoto and Paul, 1994). In *vivo* IL-4 has been shown to initiate Th2 type responses (Lu et al., 1994; Seder and Paul, 1994; Kopf et al., 1993;

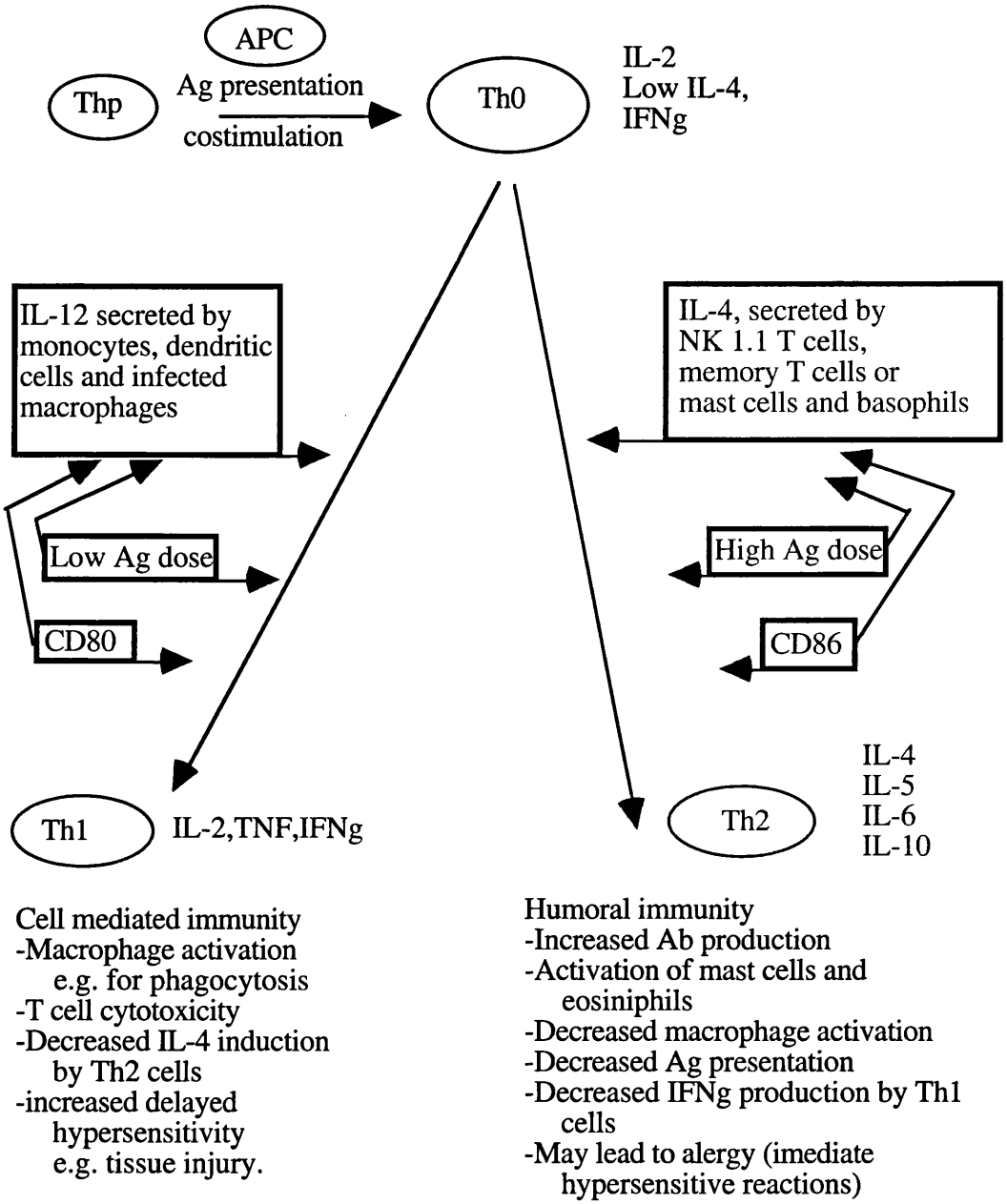

Figure 1.2: Control of Th1 and Th2 T cell differentiation. After activation T cells pass through an initial phase that is characterised by mainly the production of IL-2. After this further differentiation is suggested to take place. The types of cytokines present within the environment are thought to play a crucial role, but other factors like costimulation and antigenic strength may also participate either directly or by influencing the production of the key cytokines like IL-12 and IL-4.

Lederer et al., 1996b) but it does not seem to be important once responses have been established, since its removal at the later stages does not block Th2 cells (Yoshimoto and Paul, 1994). Considerable debate exists about the source of this IL-4, with a small population of $NK1.1^{+ve}$ T cells (Yoshimoto and Paul, 1994) and basophils / mast cells (Romagnani, 1992) being possible candidates. More recent studies with NFAT knockout mice suggested that the control of IL-4 gene expression and secretion may be regulated by the control of the transcriptional ability of NFAT proteins within the T cells (Kiani et al., 1997). Thus, sustained IL-4 production by NFAT will mediate Th2 cell generation, but in other cases a short-lived IL-4 expression will instead promote Th1 cells development (Kiani et al., 1997). Distinct composition of NFAT complexes may aid this differential regulation (Kiani et al., 1997; Yoshida et al., 1998; Ranger et al., 1998). Apart from the regulation of NFAT, the levels of STAT6 in the cells may also be a crucial control point during differentiation as others have suggested (Lederer et al., 1996b) and as the inability of $STAT6^{-/-}$ mice to mount a Th2 response has shown (Takeda et al., 1996; Shimoda et al., 1996). Overall these data suggest that the signals that control these transcription factors may play a vital role in the promotion of Th2 responses.

In the absence of IL-4, T cells differentiate in Th1 cells by default. However IL-12 is strongly implicated in the generation of Th1 cells (Lederer et al., 1996b; Seder and Paul, 1994). One of the main outcomes of IL-12 transcriptionally is the induction of the transcription factor $STAT4^{-/-}$ which is suggested to play a crucial role (Kaplan et al., 1996), but not essential (Kaplan et al., 1998), in the generation of the Th1 responses. IL-12 is thought to synergise with CD28 and promote IFNγ production by T cells (Romagnani, 1992; Peng et al., 1997) which in turn also supports Th1 cells indirectly, by limiting the growth of IL-4 producing cells (Seder and Paul, 1994).

1.1.4.4b: Effect of costimulation on T cell differentiation.

The ability of CD28 to synergise with IL-12 and induce Th1 cells may suggest that costimulation promotes Th1 cells. However, most studies have clearly suggested the ability of CD28 to preferentially promote Th2 cytokines and functions (Seder et al., 1994; De-Boer et al., 1993; Rulifson et al., 1997). Furthermore, $CD28^{-/-}$ mice seem to have their Th1 effector functions intact but their Th2 responses decreased (Shahinian et al., 1993; Ferguson et al., 1996). More recent studies with the CD28 homologous receptor CTLA-4 have suggested that this receptor is expressed at higher levels in Th2 cells, although it is able to negatively regulate T cell activation in both Th1 and Th2 cells (Alegre et al., 1998). Overall, these data support a role for costimulatory molecules in T cell differentiation, with a possible higher importance in Th2 development.

An alternative line of thought suggests that each type of response may require costimulation at different time points (Thompson, 1995). This comes from studies with genetically predisposed mice towards a Th2 response after *L. Major* infection (BALB/c) in which CTLA-4Ig is able to block Th2 responses but only when given early after infection suggesting that costimulation is required for priming of Th2 responses (Thompson, 1995; Lu et al., 1994; Murphy et al., 1997) by possibly aiding IL-4 responsiveness (McArthur and Raulet, 1993; Freeman et al., 1995). Since at the early stages of T cell activation CD86 is mainly present on the APCs, it follows that CD86 may actually promote Th2 responses (Bluestone, 1995; Freeman et al., 1995; Thompson, 1995). This is also suggested by indirect in *vivo* studies with certain Th1 mediated autoimmune diseases, like EAE. Blockade of the CD86 molecule with antibodies has actually resulted in the blockade of Th2 responses and the acceleration of the disease progress, whereas blockade of CD80 has resulted in the opposite outcome by allowing CD86 to act alone and promote protective Th2 responses (Kuchroo et al., 1995; Lenschow et al., 1995). In contrast to Th2, Th1 responses are less dependent on costimulation during their priming and need it mainly at later stages. In transplantations involving renal allografts, delayed but not

early CTLA-4Ig treatment increases survival by preventing Th1 cell generation (Sayegh et al., 1995). The fact that CD80 is upregulated during an immune response, may indicate the need of this ligand for maintaining these Th1 responses (Bluestone, 1995; Thompson, 1995). In fact, CD80 (but not CD86) transfection is able to aid tumour rejection, a Th1 mediated response. Similarly, studies in mice infected with the nematode L. *donovani* have shown that persistent blocking of CD86 (with anti-CD86) is required to mediate Th1 responses and clear the parasite (Murphy et al., 1997).

The differential role of each B7 molecule on T cell differentiation has been under considerable debate (Murphy et al., 1997; Brown et al., 1996; Schweitzer et al., 1997; Sayegh et al., 1995; Seder et al., 1994), but most probably other parameters also affect T cell differentiation. Apart from the cytokines mentioned above, the strength and type of the antigenic stimulus (Anderson et al., 1997; Liblau et al., 1996; Constant et al., 1995; Hosken et al., 1995) and other costimulatory molecule may excert pressure towards a specific T cell differentiation outcome. SLAM (Cocks et al., 1995; Aversa et al., 1997) and 4-1BB (Melero et al., 1998; Kim et al., 1998b) for example are thought to swing the action of CD28 costimulation in favour of Th1 differentiation. In contrast CD30 expressing cells may utilise CD28 in favour of Th2 outcome instead (Gilfillan et al., 1998).

It is obvious, that for the two CD28 ligands to result in different effects, they must either differ in their signalling ability through CD28, or they may utilise CD28 and / or CTLA-4 with variable affinities. Concerning the latter, CD86 dissociates faster that CD80 when bound on CTLA-4, but the significance of this difference is unknown (Linsley et al., 1994). Similarly, they have been no conclusive reports supporting differential activation of signal mediators by the two ligands (Lanier et al., 1995). However, a low level preferential induction of IL-4 and to a lesser extent TNFβ by CD86 has been observed, whereas CD80 is thought to preferentially increase IFNγ and GM-CSF (Freeman et al., 1995; Kuchroo et al., 1995) production.

Despite these differences however no intracellular signalling differences have been suggested between the two types of ligands. Some studies have suggested that CD28 can signal differently when activated by antibodies compare to CD80 or CD86, supporting the ability of the receptor to stimulate separate pathways after distinct engagement (Nunes et al., 1994). In a different study various antibodies were seen to synergise differently with PMA in terms of IL-2 production, possibly due to variable binding since not all antibodies competed each other on CD28 binding (Nunes et al., 1993).

1.2: TCR SIGNAL TRANSDUCTION.

The interaction of the MHC molecules present on APCs with the TCR on the T cells, results in at least part of the signals that the T cell requires in order to initiate an immune response. One of the first observable effects of this interaction is the increased protein tyrosine phosphorylation. The TCR has no intrinsic tyrosine kinase activity and instead utilises a number of protein tyrosine kinases that phosphorylate a large number of proteins in the cells, including the TCR itself. Immediately after that, cytoskeletal rearrangements take place and calcium fluxes are observed. Soon after, signalling cascades that involve the activation of PKC and Ras are initiated. Given the enormous and increasing complexity of this subject only an outline of the pathways involved are discussed here.

1.2.1: TCR and Protein Tyrosine kinases (PTKs).

PTKs are generally divided into membrane associated src family ($p56^{lck}$, $p59^{fyn}$ and $p60^{yes}$), cytoplasmic syk family (syk and ZAP 70) and Tec family (Btk, Itk, Tec)

kinases (Rudd et al., 1994; Bolen, 1995; August et al., 1994). All have been suggested to play a role during T cell activation but the src kinases and especially lck, are the ones that are thought to initiate the events following TCR engagement. Current evidence obtained with transformed cell lines, suggest that initially CD4 or CD8 associates with lck (Glaichenhaus et al., 1991), leading to the phosphorylation of specific motifs, called immunoreceptor tyrosine based activation motifs (ITAMs) (Rudd et al., 1994; Sancho et al., 1993; Samelson and Klausner, 1992; Glaichenhaus et al., 1991). Three such motifs are present on the ζ chain of the TCR and one is also present in the each of the CD3γ, δ and ε chains (Sancho et al., 1993; Wegener et al., 1992). The action of fyn is less well defined, but it is thought to bind CD3 and phosphorylate similar motifs (Timson-Gauen et al., 1994). The purpose of these phosphorylations, is the recruitment of ZAP70 at the ITAM motifs and its activation (Rudd et al., 1994; Chan et al., 1995). However in, "real / normal" T cells ZAP70 is suggested to be constitutively associated with phosphorylated TCRζ chain and the src PTKs may just activate it (Robey and Allison, 1995).

CD4/lck and TCR/CD3/lck/fyn/ZAP70 form a large complex whose activity directly or indirectly leads to downstream targets, such as PLCγ (Park et al., 1991; Bolen, 1995). ZAP70 dominates this downstream action (Graef et al., 1997; Elder et al., 1994). The picture is however far from complete and becomes more complicated due to the number of SH2 and SH3 domains of PTKs, which can recruit additional proteins in the complex (Rudd et al., 1994). Fyn for example is thought to recruit a p120/130 protein, now called FYB, which in turn activates SLP-76 and possibly vav, both important for IL-2 induction after engagement of the TCR (Da Silva et al., 1997). Furthermore the TCR it self is capable of interacting with other proteins after tyrosine phosphorylation. Most importantly, Ras is activated downstream of the TCR.

1.2.2: PKC induction and the calcium dependent signals of the TCR.

1.2.2.1: PKC activation via PLCγ_1.

As mentioned above non-receptor protein tyrosine kinases are responsible for the activation of phospholipase Cγ_1 (PLCγ_1) downstream of the TCR (Park et al., 1991; Bolen, 1995). As a result, PLCγ_1 breaks down phosphoinositol 4,5 bisphosphate (PIP_2), into the cyclic inositol 3,4,5 trisphosphate molecule (IP_3) and diacylglycerol (DAG). DAG remains in the membrane and acts to translocate the serine/threonine kinase PKC, from the cytosol to the inner side of the membrane (Bornancin and Parker, 1996). Furthermore DAG is thought to bind to the regulatory subunit of PKC and prevent its inhibitory action and also to increase the affinity of the enzyme for calcium. However calcium elevation also takes place in the cell after the binding of IP_3 to the IP_3 receptor on the surface of intracellular calcium stores at the endoplasmic reticulum. Calcium/calmodulin further downstream, is suggested to activate PKC even more by releasing an inhibitory factor called AKAP79 (Faux and Scott, 1997). A fully active membrane bound complex of DAG, PKC and calcium is therefore formed (Newton, 1996). Additional calcium dependent proteins are activated, with calcineurin taking a central role in TCR signalling (**figure 1.3**).

1.2.2.2: Calcium/calcineurin dependent signals of the TCR

After TCR engagement two phases of calcium fluxes follow. A transient early release from intracellular stores (after IP_3 formation) induces a prolonged later influx from outside the cell (Sei et al., 1995; Takemura et al., 1996; Premack et al., 1994; Chakrabarti et al., 1995). The coupling of the two fluxes is thought to be mediated via a mechanism that involves PTKs (Marhaba et al., 1996) and calmodulin (Haverstick et al., 1997). A sustained calcium elevation (for at least 30 minutes) is thought to be vital for proliferation and IL-2 production. Cross-linked CD3 antibodies easily achieve this but physiologically, the TCR has low affinity for MHC and a rapid off rate. Sustained calcium signalling is therefore achieved by

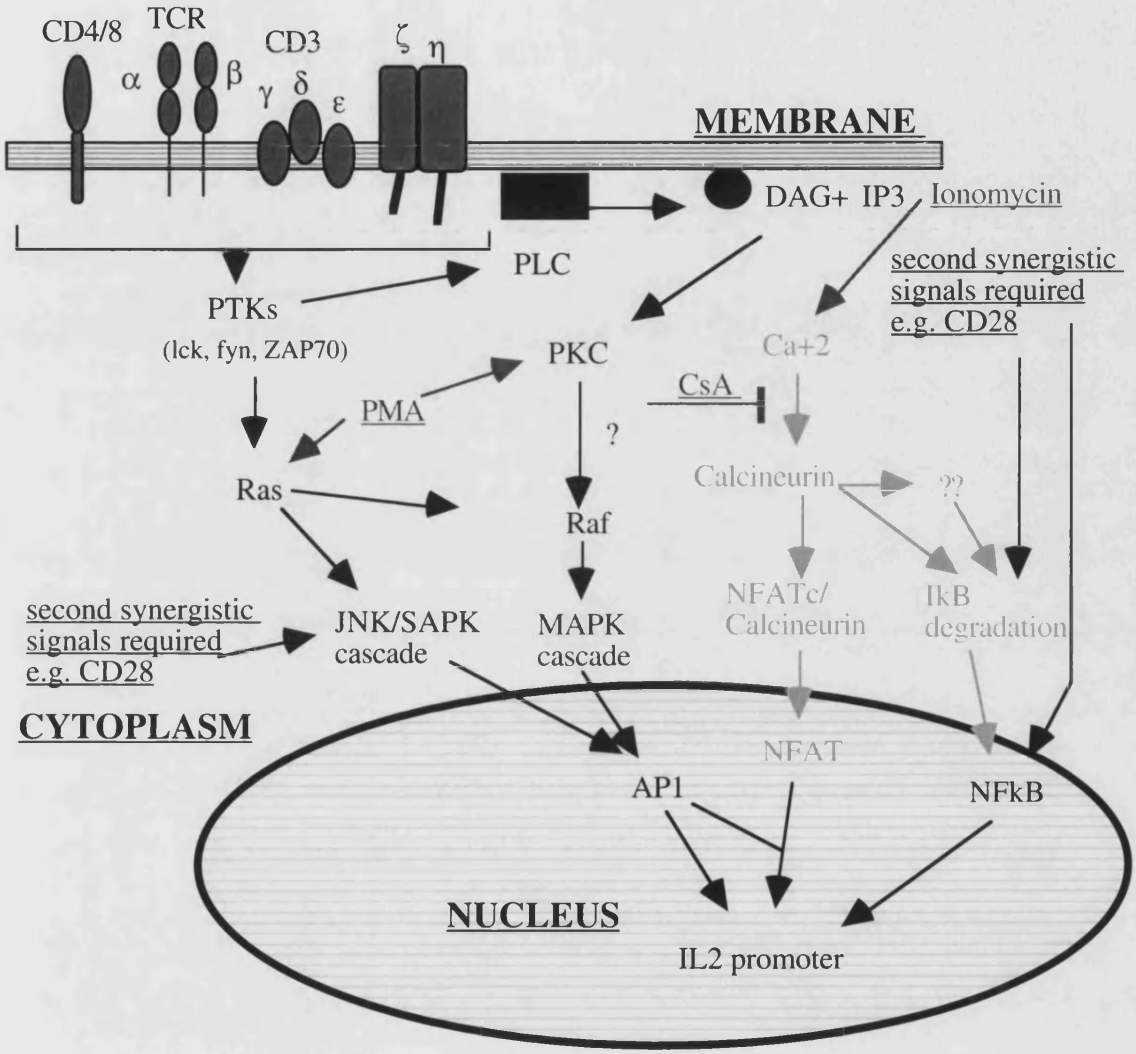

Figure 1.3: The signals initiated after TCR engagement. Engagement of the TCR/CD3 complex is followed by activation of a number of protein tyrosine kinases (PTKs) which aid the activation of PLCγ1. Further downstream the signals are divided in to calcium dependent (green) and calcium independent (blue). PTKs are also responsible for the calcium independent initiation of the Ras pathway (see section 1.2.3.1 and figure 1.5). The sites of action of PMA, ionomycin and CsA are also shown (red).

continuous use of different TCRs (Ratcliffe et al., 1992; Valtutti et al., 1995), a process referred to as receptor counting (Viola et al., 1997a). Cytoskeletal rearrangements and actin reorganisations are thought to aid that by mobilising the cell surface and allowing TCR molecules to scan on the surface of APCs (Valtutti et al., 1995; Delon et al., 1998). Interestingly the costimulatory molecule CD80 has also been suggested to bind its counter-receptors with very fast kinetics, a fact that may further accommodate the scanning for the antigen on the surface of the APC (van der Merwe et al., 1997).

The binding of four calcium molecules on calmodulin alters its conformation and this is transmitted to target proteins affecting their activity. Downstream of the TCR, a similar mechanism activates a well documented serine/threonine phosphatase called calcineurin, which is also the target of the immunosupressive drug CsA (Guerini, 1997). CsA acts as an immunosupressant in transplantation and autoimmunity. Biochemically it binds cyclophilin, a highly basic and abundant enzyme that is characterised by its peptidyl-polyl-cis-trans-isomerase activity (rotamase) (Liu, 1993). After binding of CsA, rotamase activity is lost because CsA has a structure called twisted amide that is a transition state between the substrate and product of the enzyme (Schreiber and Crabtree, 1992). However, the immunosupressive properties of CsA do not rely on this, but instead on the ability of the CsA/Cyclophilin complex to bind calmodulin and calcineurin (Liu, 1993; Schreiber and Crabtree, 1992).

An important role of calcineurin is the translocation of cytoplasmic NFAT proteins to the nucleus were they combine with AP1 proteins, to create transcriptionally active NFAT complexes. Other processes however also utilise calcineurin (**Figure 1.4**), although compared to other phosphatases its target specificity is thought to be limited (Guerini, 1997). The possible role of calcineurin on intracellular trafficking is interesting for CTLA-4, whose expression is controlled via the exocytotic/endocytotic cycle (Linsley et al., 1996; Alegre et al., 1996; Zhang and

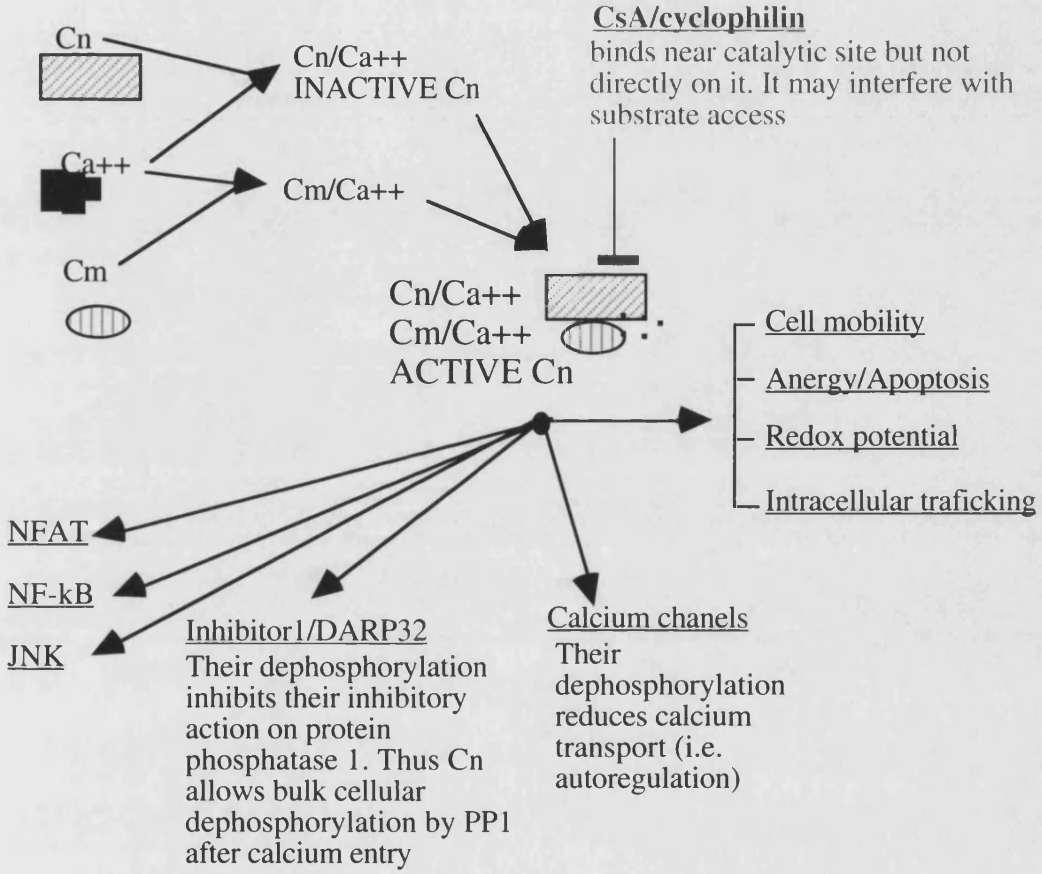

Figure 1.4: Activation and the effects of calcineurin. Calcineurin (Cn) and calmodulin (Cm) bind calcium ions and they interact together to make an active calcineurin molecule. CsA is not suggested to affect this interaction but prevent access of the substrate at the catalytic site. A number of molecular mechanisms are thought to be affected by calcineurin. but concerning its direct molecular action it is mainly known for its ability to interact with the transcription factor NFAT and translocate it to the nucleus of the cell.

Allison, 1997; Bradshaw et al., 1997; Chuang et al., 1997; Shiratori et al., 1997). In mast cells and gastric chief cells exocytosis is sensitive to CsA, whereas ocadaic acid that blocks PP1 and PP2A (other type of phosphatases) does not affect it (Raufman et al., 1997). A 55kDa cytoskeletal protein is thought to be targeted by calcineurin in these cells, suggesting that calcineurin may affect cytoskeletal proteins that aid the fusion of secretory vesicles with the plasma membrane (Raufman et al., 1997).

1.2.2.3: PMA and ionomycin.

Overall signalling from the TCR can be divided into calcium independent and calcium dependent pathways (Mueller et al., 1990; Takahama and Nakauchi, 1996). Interestingly, although these two types of signals synergise together to complete the TCR signals, they also compete with each other. Specifically, PKC activation is suggested to actually downregulate the calcium dependent signals (Cantrell et al., 1989; Haverstick et al., 1997) by decreasing the rate of calcium influx from the endoplasmic reticulum (Haverstick et al., 1997). The tumour promoting agent PMA, which amongst others, activates the serine/threonine protein kinase C (PKC), can mimic at least part of the calcium independent signals of the TCR (Takahama and Nakauchi, 1996). On the other hand the calcium signals can be induced by calcium ionophores like ionomycin (Gunter et al., 1989; Chatila et al., 1998). Despite the fact that the TCR can not activate T cells alone, PMA and ionomycin are able to result in necessary signals for T cell activation. It therefore seems that PMA and ionomycin, are such a powerful signal, that they resemble a very strong antigenic stimulus, which does not require costimulation. This is supported by the fact that stimulations resulting with low concentrations of PMA and ionomycin can be costimulated by CD28 antibodies (June et al., 1989). However it is also possible that PMA activates CD28 or other costimulatory pathways.

1.2.3: PKC, Ras and the MAPK cascade.

1.2.3.1: Activation of Ras by the TCR.

Ras and specifically $p21^{Ras}$, is a GTP binding protein, essential for TCR signalling (Baldari et al., 1993), but not sufficient by itself (Pastor et al., 1996). It is activated by guanine nucleotide exchange factors (GEFs), which promote the release of GDP and its replacement with GTP. Inactivation takes place after the induction of intrinsic GTPase activity via GTPase activating proteins (GAPs). Sos which is thought to play the role of GEF for Ras, is found associated with Grb2 in the cytoplasm via the interaction of the SH3 domain of Grb2 and the proline-rich region of Sos (Rozakis-Adcock et al., 1993; Li et al., 1993; Lowenstein et al., 1992). After receptor activation, Grb2/Sos is translocated as a complex to the membrane (Aronheim et al., 1994; Stokoe et al., 1994), via an adaptor protein that contacts the TCR at phosphotyrosine residues via its SH2 domain. Two such proteins are known to be active in T cells, namely p36 and the 55kDa shc protein (Lowenstein et al., 1992; Bork and Margolis, 1995; Rozakis-Adcock et al., 1993), both phosphorylated and activated by ZAP70. TCR activation seems to use p36, whereas shc participates in IL-2 signalling (Pastor et al., 1996). A trimeric p36/Grb2/Sos complex is therefore formed which acts as a link between TCR and Ras (Li et al., 1993; Aronheim et al., 1994) **(Figure 1.5)**. Other adaptor proteins such as cbl, PTP-1C, PTP-1D, vav and even p85 of PI3K also interact with Grb2 but their specific role in TCR signalling is still unclear (Bork and Margolis, 1995; Kim et al., 1998a).

Ras activity is suggested to be regulated by the activation of GAPs which inhibit Ras. In fact, PMA is suggested to inhibit such proteins and therefore allow Ras to act (Gulbins et al., 1994). It is not clear however if this effect is direct on GAPs or to other proteins that associate with them (Hall, 1996; Downward et al., 1992). Rap1 is an alternative regulator of Ras activity that acts by sequestering effector molecules of Ras and therefore limiting the pool on which Ras initiates its functions. Since Rap1 has been found to be elevated in anergic cells it may partially explain the

inability of Ras to act in anergic cells (Reedquist and Bos, 1998). Further studies, have shown that anergic T cells stimulated with CD3 and CD28, are incapable of accumulating active Ras-GTP (Fields et al., 1996) and are characterised by reduced levels of MAPK, AP1 and NFAT compared to control cells (Fields et al., 1996; Carmella et al., 1996). However PMA and ionomycin which synergise and rescue cells from this anergic state can also activate Ras, suggesting that the blockade that mediates anergy must lie upstream, in the link between Ras and TCRζ (**figure 1.5**). It was indeed found that after TCR stimulation Sos was recruited to the membrane, but was not phosphorylated as it was in control cells. Others associations and phosphorylations were as normal (Fields et al., 1996). These studies however examined the association of Grb2/Sos with the adaptor protein shc which, as discussed above, may not be the main protein involved after TCR stimulation (Pastor et al., 1996). Furthermore, evidence suggests that Sos may not require phosphorylation for its activation (Aronheim et al., 1994).

1.2.3.2: The Raf-MAPK pathways.

The ability of PMA to activate PKC and Ras suggested initially that PKC is a downstream target of Ras. Despite this, not all receptors that activate Ras also activate PKC (Downward et al., 1992) and inhibition of PKC does not abolish the ability of Ras to synergise with ionomycin and induce IL-2 (Williams et al., 1995). It is therefore more likely that PKC and Ras are involved in separate and parallel pathways, activating similar targets. Such a target is the serine/threonine protein kinase Raf-1 (or MAPKKK or MEKK), which initiates the mitogen activated protein (MAP) kinase cascade (Warne et al., 1993; Ruderman, 1993; Kyriakis et al., 1992) (**Figure 1.3 and 1.5**). Raf-1 is essential for TCR signalling, but it is not able to synergise with calcium and produce IL-2, clearly showing that more downstream effectors of Ras are also involved (Kyriakis et al., 1992; Owaki et al., 1993). The N-terminal of Raf-1, binds Ras-GTP and as a result is translocated to the membrane (Warne et al., 1993; Stokoe et al., 1994), where it is activated by both serine/threonine and tyrosine phosphorylation, possibly aided by PKC and PTKs

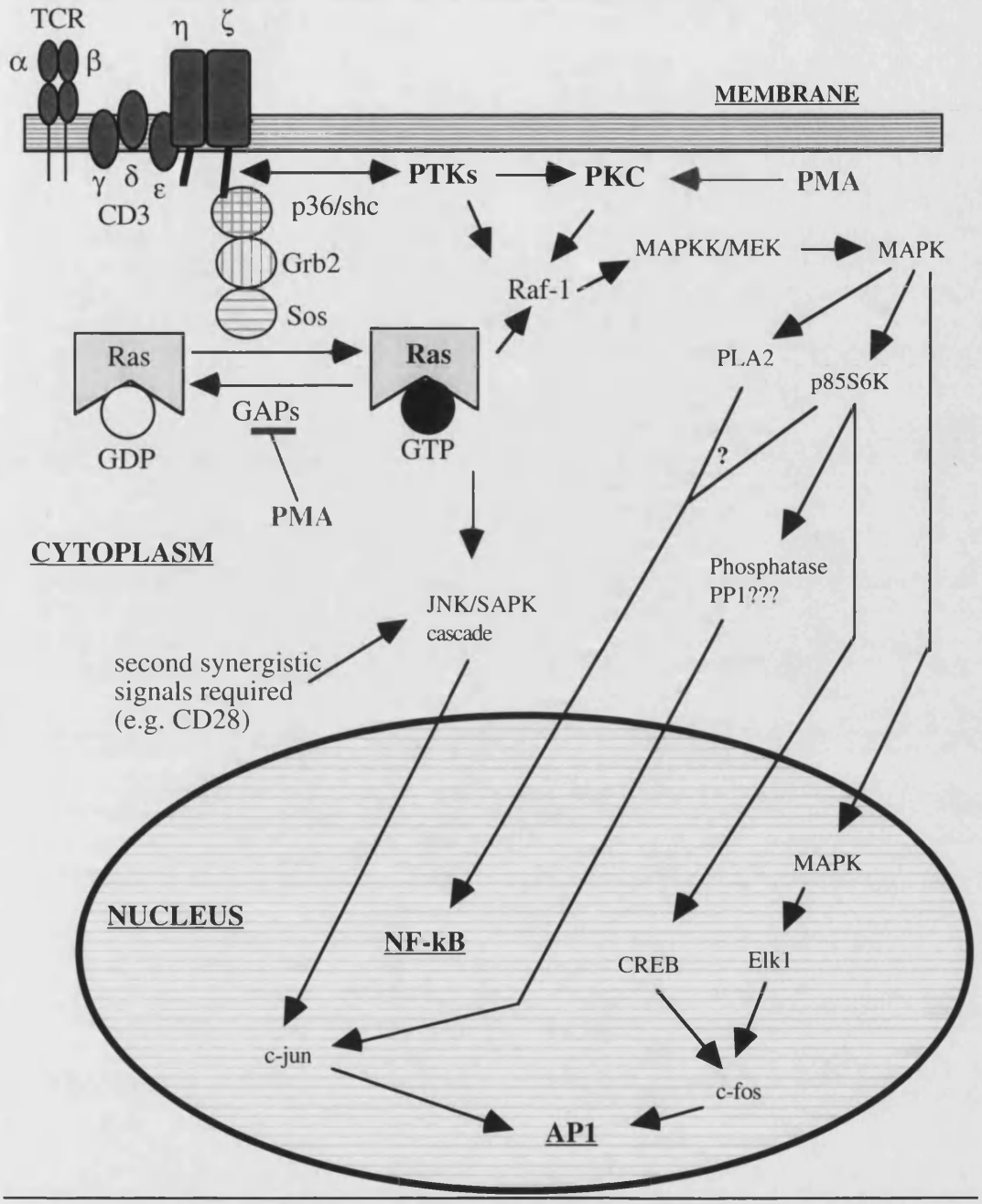

Figure 1.5: The Ras-MAPK pathway downstream of the TCR. PTKs are important for the initiation of the Ras pathway since certain tyrosine residues on the TCR cytoplasmic tails need to be phosphorylated. These act as docking sites for SH2 containing proteins (e.g. p36 or shc) which ultimately link the TCR with Ras (see text for details). Ras is a central molecule downstream of the TCR and is thought to mediate a number of effector functions. Most importantly, Ras translocates Raf1 to the membrane, which is the first kinase in the MAPK cascade and its downstream targets (see text for details).

respectively, both of which are present in the membrane after T cell activation (Stokoe et al., 1994). Downstream of Raf is MAPK kinase (MAPKK or MEK) which is a dual specificity kinase, phosphorylating the final kinase of the pathway MAPK at both threonine and tyrosine on a TEY motif (Kyriakis et al., 1992). Two types of MAPK are known, called ERK1 (44kDa) and ERK2 (42kDa) which are generally activated by mitogens and PMA (Ahn et al., 1992; Ruderman, 1993). Both are proline directed serine/threonine protein kinases, phosphorylating sites with the sequence PXS/TP, were X can be any amino acid. Cytoplasmic phospholipase A_2 (PLA_2), which also needs calcium for its translocation to the membrane, is one substrate of ERKs (Lin et al., 1993) **(figure 1.5)**. Another possible target of MAPK, p85S6 kinase, (now also called MAPK-activating protein kinase 1 or MAPK-APK1), is translocated in the nucleus after activation (Xing et al., 1996; Rouse et al., 1994) and has been suggested to participate in the activation of NF-kB by acting with other kinases and aiding the phosphorylation and degradation of IkBα (Kanno and Siebenlist, 1996; Ghoda et al., 1997). It is however recognised more as the activator of CREB (cAMP responsive element binding protein), that is required for c-fos transcription (Xing et al., 1996).

Apart from acting in the cytosol, MAPK can be translocated in the nucleus **(figure 1.5)**. Its primary target seems to be $p62^{TF}$ (Elk1), which is constitutively present on the serum response element (SRE) and is activated transcriptionally by serine phosphorylation at the C terminus and together with the serum response factor (SRF) it activates c-fos transcription (Gille et al., 1993). c-fos belongs to the fos family of AP1 transcription factors but can not make up an active dimeric AP1 transcription factor by itself. Instead it needs a member of the jun family, of which c-jun is the most important (Karin, 1995). MAPKs have been suggested to participate in c-jun activation, but the mechanism is unclear. A protein phosphatase, similar to PP1 activated by p85S6 kinase in skeletal muscle (Rouse et al., 1994; Ruderman, 1993), may play a role by dephosphorylating specific serine residues required for c-jun activation. Alternatively MAPK may also increase AP1

transcriptional activity indirectly by favouring c-jun/c-fos heterodimers from c-jun homodimers which are less active. In this respect MAPKs are suggested to phosphorylate the DNA binding region of c-jun and reduce its DNA binding activity only when they are present as homodimers. In any case it must be noted that although dominant active Ras has been shown to induce high AP1 activity (Binetry et al., 1991), dominant active MAPKK (MEK) is unable to do so (Karin, 1995, Genot et al., 1996). The effect of MAPK on c-jun would therefore not be enough for full activation. Instead, a similar cascade to MAPK, called JNK (c-jun N-terminal kinase) is thought to be involved (Minden et al., 1994; Hibi et al., 1993). Generally in contrast to the MAPK cascade, CD3 antibodies, PMA and Ras can only stimulate this pathway at low levels (Derijard et al., 1994; Smeal et al., 1991; Binetry et al., 1991; Minden et al., 1994). A second signal is also needed which it is possibly mediated by Rac-1 and Cdc42 after CD28 signalling making JNK pathway an integration point for TCR and CD28 signalling (Su et al., 1994; Faris et al., 1996; Jacinto et al., 1998).

1.3: CD28 SIGNALLING.

The signals initiated by CD28 are independent of but cooperate with TCR signals for full T cell activation. This is clearly seen by the differential sensitivity to cyclosporin A which blocks TCR signalling (Emmel et al., 1989) but leaves some if not all CD28 downstream pathways intact. Also distinguishing the two receptors is the different pattern of PTK induced tyrosine phosphorylation, observed after stimulation (Vandenburghe et al., 1992; Boussiotis et al., 1996; Hutchcroft et al., 1996; Lu et al., 1992). Generally however, CD28 induced signals synergise with TCR and allow the full repertoire of transcription factors, including NFAT, AP1 and

NF-kB, to be generated, activated or translocated in the nucleus (Durand et al., 1988, Go and Miller, 1992; Fraser and Weiss, 1992; Garrity et al., 1994). As a result the proliferative cytokine IL-2 (Fraser et al., 1991; Granelli-Piperno and Nolan, 1991; Hughes and Pober, 1996), but also IL-3, GM-CSF, IFNγ, TNF, IL-5 (Gimmi et al., 1991; Thompson et al., 1993; Reiser et al., 1992) as well as IL-4 (Seder et al., 1994), IL-8 (Weschler et al., 1994), IL-10 (Peng et al., 1997) and IL-13 (Minty et al., 1993) are expressed. Additionally CD28 is suggested to promote the stability of cytokine mRNAs by possibly preventing the degradation of AU rich sequences (Lindsten et al., 1989; Umlauf et al., 1995; Cerdan et al., 1992). All these factors seem to suggest the signalling importance of CD28 on T cell activation.

1.3.1: The Phosphoinositide-3 kinase (PI3K) pathway.

1.3.1.1: Activation and structure of PI3K.

CD28 engagement is thought to result in the phosphorylation of at least four tyrosine (Y) residues on the cytoplasmic tail of CD28 which are then used for recruiting various proteins (Lu et al., 1992; King et al., 1997). The best documented is the recruitment of PI3K at Y_{173} of the highly conserved YMNM motif (Prasad et al., 1994; Truitt et al., 1994; Stein et al., 1994). PTKs perform a crucial role in this association by phosphorylating Y_{173} and PI3K. Typical src PTKs, (lck and fyn) possibly participate, although they are not thought to be essential (Gibson et al., 1998; King et al., 1997; Raab et al., 1995). Signals from both the CD28 and the TCR receptor are thought to synergise and phosphorylate CD28 (Raab et al., 1995). In fact, in the presence of TCR signals, CD28 is thought to activate a PTK called $p72^{ITK}$ (inducible T cell kinase) that may synergise with ZAP70 and phosphorylate CD28 at four tyrosine residues (Gibson et al., 1998; Marengere et al., 1997; August et al., 1994; King et al., 1997). This is still controversial however, since $Itk^{-/-}$ cells were found to proliferate and produce more IL-2 after TCR (or PMA) and CD28 stimulation (Liao et al., 1997).

Structurally, PI3K is a heterodimer consisting of an adaptor p85 and a catalytic p110 subunit (Hiles et al., 1992; Ward et al., 1996). The p85 subunit contains a C-terminal and an N-terminal SH2 domain, both supporting intermolecular interactions with pY_{173} of CD28. Amongst other regions, it also contains one SH3 domain which allows associations with other proteins containing a pro-rich region, including CD28 itself (Pages et al., 1996). Two proline-rich regions are also present in PI3K and are used by the SH3 domains of PTKs, to phosphorylate, translocate to CD28 and regulate PI3K activity. Finally a p110 binding site is present, which links the CD28 and PTKs to the catalytic p110 subunit of PI3K (Hiles et al., 1992).

p110 does not participate in the interaction with CD28, but phosphorylates the D3 position of the inositol in phosphatidylinositols, giving rise to D-3-phosphoinositides and in particular PIP_3 (phosphatidylinositol-(3,4,5)-trisphosphate) (Ward et al., 1993; Truitt et al., 1994). Wortmannin blocks the activity of PI3K by binding to the ATP binding site of the p110 catalytic subunit and hence reducing the production of D-3-phosphoinositides, but does not affect the association of PI3K with CD28 however (Yano et al., 1993; Ward et al., 1995; Stephens et al., 1991). Other studies with the insulin receptor have also suggested that the p110 subunit is able to phosphorylate serine residues of other proteins and itself in a wortmannin sensitive manner (Dhand et al., 1994; Hunter, 1995; Lam et al., 1994). However, although ligation of CD28 via CD80 in Jurkats leads to serine/threonine phosphorylation of CD28, neither PKC nor PI3K have been found responsible, suggesting a role for an unknown kinase (Parry et al., 1997).

1.3.1.1: Downstream targets and functions of PI3K.

PI3K recruitment and activity (Ward et al., 1995; Prasad et al., 1994; Truitt et al., 1994; Pages et al., 1994) are suggested to play an important role downstream of CD28. From the D3-phosphoinositides produced by PI3K, phosphatidyl-3,4,5-trisphosphate (PIP_3) is suggested to be the most important product (Berridge and Irvine, 1984). Other mitogenic receptors (e.g. PDGF) use PI3K and these lipids for

their proliferating / mitogenic signals, thus suggesting that PI3K may promote T cell growth after CD28 engagement. This is also suggested by one of the downstream targets of PI3K, protein kinase B (PKB), which is a homologue of the transforming protein v-act (Franke et al., 1995; Burgering and Coffer, 1995; Parry et al., 1997). D3-phosphoinositides and especially PIP_3, may activate PKB directly by interacting with its PH (plecstrin homology) domain (Franke et al., 1995; Hemmings, 1997). This allows the translocation of the kinase to the membrane, where it is thought to be phosphorylated and activated by a constitutive serine/threonine kinase (Hemmings, 1997). Further downstream PKB is thought to activate p70S6 kinase (Ericson, 1991; Hemmings, 1997; Pai et al., 1994; Chung et al., 1994), via a not so well characterised enzyme that is called target of rapamycin, TOR, because its activity is directly blocked by the drug/complex rapamycin/FK506-Binding Protein (FKB) (Chung et al., 1992; Price et al., 1992; Kunz et al., 1993) **(figure 1.6)**. Several studies have been involved with the identification of the target of rapamycin. TOR1 and TOR2 in yeast and mTOR in mammals, have serine/threonine kinase and PI4K activity and are suggested to be essential for growth (Kunz et al., 1993). Interestingly these TOR molecules are related to PI3K and are even inhibited by wortmannin in some cases (Sabatini et al., 1994; Brunn et al., 1996). In T cells, a protein called FRAP (FK506-binding protein Rapamycin Associated Protein) interacts with the rapamycin/FKBP complex and is thought to act as TOR (Choi et al., 1996).

The target of TOR, p70S6 kinase, phosphorylates the S6 ribosomal protein, but since rapamycin has been suggested to prevent the degradation of IkBα via CD28 (Lai and Tan, 1994), TOR and possibly p70S6 kinase may be involved in the regulation of the NF-kB transcription factor. Another molecule that has been seen to be activated directly by PIP_3 *in vitro* is the atypical ζ isoform of PKC, which is insensitive to both phorbol esters and calcium (Nakanishi et al., 1993). The acidic phospholipid ceramide, that is induced by CD28 may also synergise with PIP_3 to activate PKCζ (Lozano et al., 1994). Overall PKCζ has been attributed survival

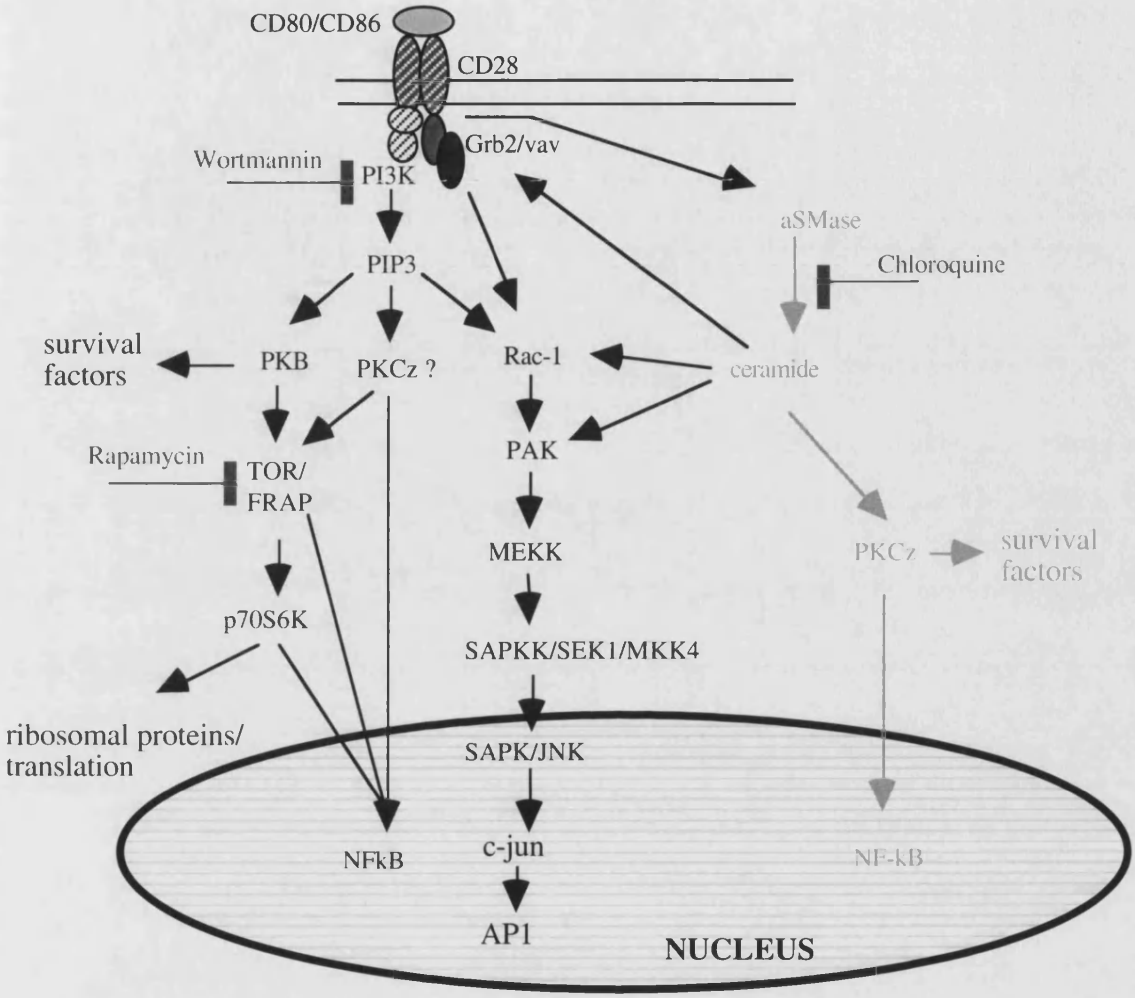

Figure 1.6: The PI3K, JNK and the predicted aSMase pathways downstream of CD28. After CD28 engagement, the cytoplasmic tail of the receptor associates with PI3K and Grb2. Downstream of PI3K certain signals are induced (blue) as detailed in the text. Grb2 interacts with vav which is thought to activate Rac and the JNK cascade (black). The link between CD28 and aSMase (green) is less clear. Overall the signals are thought to target transcription factors that participate in the activation of certain genes including IL-2. Survival factors that are also induced include the activation of bcl-X_L. The sites of action of wortmannin, rapamycin and chloroquine are also shown (red).

functions (Diaz-Meco et al., 1996) and is also suggested to activate the transcription factor NF-kB (Muller et al., 1995; Lozano et al., 1994), however the enzyme remains poorly characterised.

Recently the role of PI3K in costimulation and IL-2 production has been questioned (Crooks et al., 1995; Collette et al., 1997; Truit et al., 1995). Although wortmannin can decrease proliferation and IL-2 production in resting T cells, the effect is less obvious in activated T cells (Edmead et al., 1996) and absent in jurkat T cells (Ueda et al., 1995). In fact some jurkats have shown increased IL-2 secretion in the presence of wortmannin, despite a decrease in the accumulation of D-3-phosphoinositides (Ueda et al., 1995). Furthermore the phorbol ester PMA can block PI3K recruitment to CD28, without interfering with IL-2 production or proliferation (Hutchcroft et al., 1995; Parry et al., 1996). It therefore seems that PI3K may be less important in active T cells. This may be because metabolically active cells are able to synthesise new PI3K *de novo* which may not be affected by the low stability wortmannin (Yano et al., 1993).

The above data suggest that PI3K may have additional roles other than mediating IL-2 production. PI3K may for example participate in T cell activation by aiding cell cycle progression and survival mechanisms in the cells and several studies support this idea. The kinase p70S6 kinase is involved in cell cycle progression, and its activation is inhibited by rapamycin, a drug that is known to lead to a G1 cell cycle arrest (Maurice et al., 1993). Many PI3K homologues are involved in cell cycle in yeast and some are essential for growth and are also sensitive to rapamycin (Hunter, 1995; Kunz et al., 1993; Brunn et al., 1996). Other T cell receptors (e.g. CD3, IL-2R) using PI3K are also responsible for cell cycle progression (Ward et al., 1992; Shibuya et al., 1992; Remillard et al., 1991). In support of the cell survival role of PI3K, the anti-apoptotic effects of nerve (Yao and Cooper, 1995; Hemmings, 1997), and insulin (Kauffman-Zeh et al., 1997) growth factor receptor is decreased by wortmannin. Additionally c-myc induced apoptosis was delayed by an active PI3K

in a wortmannin sensitive manner (Kauffman-Zeh et al., 1997). Finally Ras signalling has been found to induce apoptotic pathways, but protective signals when PI3K and PKB are induced (Kauffman-Zeh et al., 1997). More importantly CD28 has been found to enhance the expression of the survival protein bcl-X_L (Boise et al., 1995b; Noel et al., 1996; Sperling et al., 1996; Levine et al., 1997). The intermediate signals in this pathway are under investigation but recent studies suggest that PI3K uses PKB, but not p70S6 kinase to promote its anti-apoptotic effects (Kauffman-Zeh et al., 1997; Hemmings, 1997). In addition signals initiated by PI3K and ceramide (see later) may synergise after CD28 engagement and enhance the activation of NF-kB, a transcription factor that is thought to promote T cell survival (Beg and Baltimore, 1996).

Recently the Rho-like GTP binding protein Rac-1, has also been described as another downstream target of PI3K (Hawkins et al., 1995), mediated by PIP_3. Rac-1 has been suggested to mediate the activation of the JNK downstream of PI3K **(figure 1.6)** (Lopez-Ilasaca et al., 1997; Coso et al., 1995; Minden et al., 1995; Jacinto et al., 1998), but it is interesting that Rac-1 and other Rho like proteins play crucial roles in the reorganisation of the cytoskeleton (Lamarche et al., 1996; Ridley and Hall, 1992; Ridley et al., 1992), an additional function that PI3K may also control. In fact a number of studies have indicated the ability of CD28 to phosphorylate and activate vav (Kim et al., 1998a; Nunes et al., 1994) which is thought to act as a GEF for the Rho-like GTP binding protein, Rac-1. Even more striking, vav is associated with Grb2, a protein that interacts with CD28 at the same motif as PI3K (Kim et al., 1998a; Prasad et al., 1994). These results suggest that PI3K may play a role on the activation of vav and subsequently Rac-1 and regulate the formation of actin stress fibres. In fact recent studies have suggested that Rac-1 and Cdc42 may perform such a function downstream of CD28 and polymerise actin at the contact site between T cells and CHO-CD86 cells. As a result the membrane is characterised by ruffles (Kaga et al., 1998a).

The importance of PI3K in cytoskeletal rearrangements is also seen by the fact that the yeast PI3K homologue Vsp34, is thought to be important for protein targeting / trafficking and vesicular transport mechanisms that require a mobile cytoskeleton. Similarly, inhibition of PI3K activity has been shown to prevent histamine secretion in basophils (Yano et al., 1993) and respiratory burst in neutrophils, processes that require protein trafficking in the cells. Finally a mutant form of PDGF that can not bind PI3K, fails to be internalised (Wennstrom et al., 1994). In connection with this, internalisation of CD28 itself has also been shown to require PI3K. About 30% of CD28 is partly degraded and / or recycled after internalisation and mutations preventing PI3K binding, decrease internalisation (Cefai et al., 1998). However it is not clear yet if PI3K enzymatic activity is also required for CD28 internalisation.

1.3.2: The acidic sphingomyelinase (aSMase) pathway.

Sphingomyelinases, are phospholipases that hydrolyse the complex sphingolipid, sphingomyelin (SM), into ceramide and phosphatidylcholine (PC) and are divided into three types depending on their localisation (Hannun, 1994; Wiegmann et al., 1994; Hannun, 1996). Two cytosolic neutral forms (one free and one membrane bound) which are Mg dependent (neutral -nSMase), and one acidic form which is Mg independent and is present in the lysosomes (acidic -aSMase) (Hannun, 1994). From the two products, ceramide has been involved in cellular processes as diverse as, differentiation, cell growth, inflammation, protein trafficking and even cell cycle arrest and apoptosis (Kolesnick and Fuks, 1995; Hannun, 1996; Pushkareva et al., 1995; Jarvis et al., 1994; Obeid et al., 1993). The topology (Wiegmann et al., 1994) together with the strength and extent (Boland et al., 1996; Hannun, 1996) of SMase activation, may determine the final outcome but at the same time the *de novo* biosynthesis of ceramide may also play a role in the strength and outcome of the signal (Hannun, 1996; Boesen-de Cock et al., 1998; Boland et al., 1996). Additionally, other aSMase products (Merrill et al., 1996) may be important since in many cases aSMase is found to be more active than ceramide alone (Higuchi et al.,

1996). The importance of aSMase in T cells is clearly seen in aSMase$^{-/-}$ mice which show reduced although not abrogated T cell proliferation compared to control mice (Stoffel et al., 1998). Interestingly IL-2 intracellular levels were higher but secreted IL-2 was lower suggesting that aSMase deficient cells may have defects in the secretion / exocytosis pathway (Stoffel et al., 1998).

Several death receptors including TNFα and Fas (Kolesnick and Golde, 1994; Hannun, 1996; Higuchi et al., 1996; Wiegmann et al., 1994) can activate SMase and use ceramide as the mediator of their functions. Stress responses like UV light are also able to activate this enzyme (Hannun, 1994; Kolesnick and Golde, 1994). TNFα is characterised by the fact that it can activate both the acidic and the neutral SMase, via distinct regions of its 55 kDa subunit and with distinct kinetics (Wiegmann et al., 1994). CD28 was recently found to activate the acidic but not the neutral form of the enzyme (Boucher et al., 1995; Edmead et al., 1996). Although SMase and ceramide signalling studies, following CD28 engagement have not been extensively performed, a picture of the possible pathways involved is emerging mainly via the use of TNFα and UV stress response. **Figure 1.6** shows some of these pathways that may be mediated by aSMase, downstream of CD28.

The most documented target of ceramide is a 97 kDa serine/threonine proline directed protein kinase (Mathias et al., 1991; Liu et al., 1994). This ceramide activated protein kinase (CAPK) has been found to phosphorylate Raf-1 and initiate the MAPK cascade (Kolesnick and Golde, 1994; Huwiller et al., 1996; Yao et al., 1995). This pathway is however thought to be initiated by the neutral form of SMase (Wiegmann et al., 1994) which is not induced after CD28 engagement. In contrast aSMase and ceramide have been shown to induce the JNK/SAPK pathway (Westwick et al., 1995; Kyriakis et al., 1994). It may achieve this by directly activating a kinase of the JNK/SAPK cascade or it may even act further upstream and activate vav which regulates Rac-1 activity and subsequently PAK and the JNK/SAPK cascade (Manser et al., 1994; Coso et al., 1995; Minden et al., 1995)

(**see figure 1.6**). Another downstream target of aSMase and ceramide is suggested to be the atypical form of PKC, PKCζ (Lozano et al., 1994) which is thought to aid NF-kB activation and the promotion of other survival factors (Diaz-Meco et al., 1996).

1.3.3: CD28 and the JNK/SAPK cascade

The c-jun kinase (JNK/SAPK) belongs to the same family of proline directed serine/threonine kinases as MAPK and is activated by inhibitors of protein synthesis (e.g. anisomycin) and other stress signals (e.g. UV light, osmolarity, heat shock, TNF), hence its alternative name stress activated protein kinase (SAPK) (Verhei et al., 1996; Derijard et al., 1994; Kyriakis et al., 1994; Gomez del Arco et al., 1996). It is the target of a cascade that involves the sequential induction of kinases, needing both serine/threonine and tyrosine phosphorylation for their activation (Kyriakis et al., 1991) (**see figure 1.6**). Two major SAPKs are known (JNK1-46kDa and JNK2-55kDa) which are similarly activated and have the c-jun transcription factor as their ultimate target (Hibi et al., 1993; Sanchez et al., 1994). JNK1, binds c-jun directly with high affinity (Hibi et al., 1993; Kallunki et al., 1996) and phosphorylates its transactivation domain at ser-63 and ser-73 (Derijard et al., 1994; Smeal et al., 1991; Binetry et al., 1991; Pulverer et al., 1991). However for complete activation, certain residues of the c-jun DNA binding C-terminal domain (ser-243, ser-249 and thr-231), must be dephosphorylated (Pulverer et al., 1991; Karin, 1995). The initial phosphorylation of ser-249 and thr-231 is performed by the constitutive casein kinase II, a nuclear serine/threonine kinase (Lin et al., 1992). A second unidentified kinase is responsible for the third site which is also suggested to have an important regulatory role since its mutation abolishes phosphorylation in the other sites as well. In both cases however a phosphatase must be activated to stop the action of these constitutive enzymes. It is interesting that, whereas PMA and Ras by themselves can not activate JNK fully, they can dephosphorylate these sites and induce AP1 activity. This phosphatase activity may therefore be a result of TCR

signalling and may explain the partial activation, seen by such stimuli. Candidate phosphatases are PP1 and PP2A, although calcineurin may also act and therefore explain the effect of CsA on AP1 (Su et al., 1994).

In T cells, CD28 is vital for the full activation of JNK/SAPK (Su et al., 1994) and the appropriate signals may be mediated by Rac-1 and Cdc42 and possibly the activation the serine/threonine kinase PAK (Coso et al., 1995; Minden et al., 1995; Kaga et al., 1998b; Manser et al., 1994). This synergy of signals required for the activation of JNK seems unique to T lymphocytes (Faris et al., 1996; Jacinto et al., 1998; Rincon and Flavell, 1994; Su et al., 1994). The point of integration is still unclear, but it may involve any of the kinases in the JNK/SAPK cascade. However, synergy could also take place further upstream, like at the activation of Rac (Faris et al., 1996; Jacinto et al., 1998; Kaga et al., 1998b). In this respect, Ras activation by the TCR has been suggested to target Rac amongst others (Faris et al., 1996; Genot et al., 1996; Osada et al., 1997).

JNK is now known to be only one member of a series a stress activated protein kinases (Derijard et al., 1994; Guenda, 1996; Kyriakis et al., 1994). JNK differs however because it is not solely associated with the induction of apoptosis. This dual effect is surprising because the transcription factor c-jun that is activated by JNK/SAPK is important for the induction of several proliferative genes including IL-2 (Su et al., 1994; Faris et al., 1996). It seems however that the additional factors that combine with c-jun to make AP1 or subsequently NFAT may determine the effect of the resulting transcription factor and its specificity. In this respect, JNK also targets other transcription factors of the jun family (junB, junD, ATF2), not all of which are thought to positively regulate transcription (Karin, 1995; Jain et al., 1995). It has also been suggested that the duration and strength of JNK activation may determine whether it is acting as a proliferative or an apoptotic signal (Chen, 1996). Studies with UV light have shown that persistent and strong induction of JNK is needed for DNA fragmentation to result. In contrast a rapid and transient

induction of JNK does not lead to death, but promotes proliferation (Chen, 1996). The signals initiated by CD28 may therefore induce a transient induction of JNK and therefore promote proliferation and survival. In contrast signals initiated by the death receptor Fas (CD95) or radiation on the other hand induce JNK persistently (Chen et al., 1996). The duration of JNK activation may be controlled and decreased by phosphatases induced by mitogenic signals but not by apoptotic signals. As a result the latter will allow JNK to act for longer times and promote death (Chen, 1996; Gomez del Arco et al., 1996).

1.4: CTLA-4 SIGNALLING

The CD28 homologue CTLA-4 is suggested to negatively regulate T cell activation by antagonising the TCR and / or CD28 signals. It is unclear however how this takes place. One possibility is that CTLA-4 binds the costimulatory ligands CD80 and CD86 and thus reduces their availability for CD28. However the fact that antibodies for the receptor can also mediate this negative activation (Krummel and Allison, 1996; Chambers et al., 1996; Walunas et al., 1996a) suggests that CTLA-4 performs a more active role. The ability of CTLA-4 to bind PI3K via a YVKM motif similar to the one that is found on CD28 suggests that some signals may be mediated by this lipid kinase (Schneider et al., 1995) but despite the recruitment, it is not clear if PI3K enzymatic activity is also required. It is in fact possible that CTLA-4 acts by sequestering PI3K and reducing the availability for CD28. However, due to its variable domains, PI3K may also act as an adaptor protein that aids interactions with other proteins (Hiles et al., 1992; Ward et al., 1996).

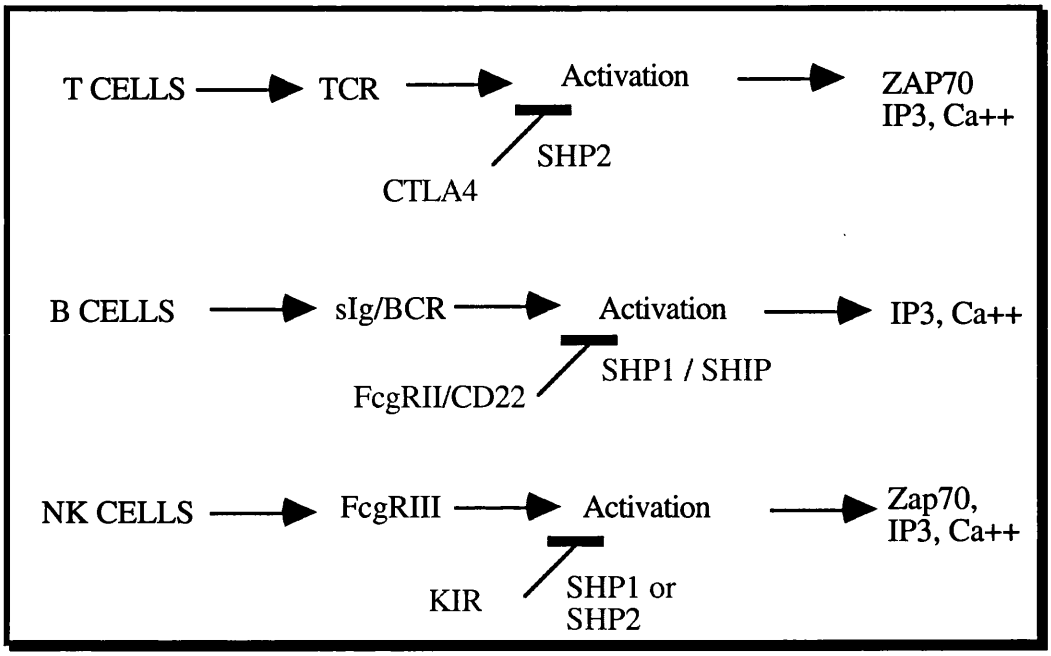

Figure 1.7: Comparison of negative regulatory signals induced by CTLA-4 in T cells with similar pathways in other cells.

More insights about the signalling abilities of CTLA-4 were obtained by the CTLA-$4^{-/-}$ mice, which are characterised by constitutively active src PTKs (lck, fyn, Zap70) as well as Ras pathway (Marengere et al., 1996). However tyrosine phosphorylation is generally increased in various proteins (e.g. TCRζ, p52Shc) and thus it is not clear if this is a consequence of the general T cell activation observed in $CTLA^{-/-}$ mice or an effect that the particular absence of CTLA-4 causes directly. Interestingly however, an SH2 containing tyrosine phosphatase called p72SYP (or SHP2 (Tonks and Neel, 1996)) is not induced in CTLA-$4^{-/-}$ mice (Marengere et al., 1996). The report suggested that the inactivation of this phosphatase in $CTLA^{-/-}$ mice may explain the constitutive activation of the above molecules and pathways. Furthermore, since SHP2 is also known to interact with the CTLA-4 cytoplasmic domain, it may mediate some of the downregulatory signals from the CTLA-4 receptor. Parallel studies in different cell types have shown similar mechanisms of negative regulation of other signals (**figure 1.7**). In B cells for example, BCR activation phosphorylates CD22 which then recruits SHP1, which is in turn able to act as a negative regulator (Nishizumi et al., 1998). SHP1 deficiency results in hyperactivation in mice (Scharenberg and Kinet, 1996). Similarly activation of NK

cells by FcγRIII is also negatively regulated by SHP1 (Scharenberg and Kinet, 1996). In both cases these receptors mediate signals very similar to the ones downstream of TCR (i.e. calcium and / or ZAP70) and the activation of the negatively regulatory phosphatase is mediated by another second receptor on the cells.

In contrast to the above observations, more recent studies have suggested that CTLA-4 is not able to decrease the levels of tyrosine phosphorylation of TCRζ or ZAP70 (Calvo et al., 1997). The same study however, clearly showed the ability of CTLA-4 to decrease ERK2 and JNK activation induced by CD3 alone or CD3+CD28 on preactivated T cells. Thus, CTLA-4 is suggested to actively block TCR signals further downstream than ZAP70 but before the level of ERK2 activation (Calvo et al., 1997).

1.5: THE IL-2 GENE PROMOTER.

The promoter of the IL-2 gene expands 5' for around 300 base pairs and contains several transcription factors binding sites as summarised in **figure 1.8**. Overall the sequences of these sites are non-canonical (i.e. not optimal, 1-2 bases from consensus) and as a result certain mutations can actually improve transcriptional activity (Hentsch et al., 1992). This non-canonical arrangement together with the presence of lymphoid specific transcription factors may allow a fine tuning of IL-2 transcription in T cells (Hentsch et al., 1992). Furthermore, some transcription factors have more than one potential binding site. The most abundant site is NFAT which although it was initially thought to be represented by two sites, three more have been reported (denoted by an asterisk in figure 1.8) (Rooney et al., 1995).

Interestingly, two of them are not novel, but are the previously characterised AP1 proximal and CD28 response element. It is not clear why some elements are present more than once, but it might have to do with formation of a higher order structure of the promoter and the regulatory proteins during activation (Rooney et al., 1995). Alternatively, multiple binding sites may be used for the regulation of some transcription factors activity depending on the amount of it present. NFAT for example has been seen to need a threshold concentration in order to be active.

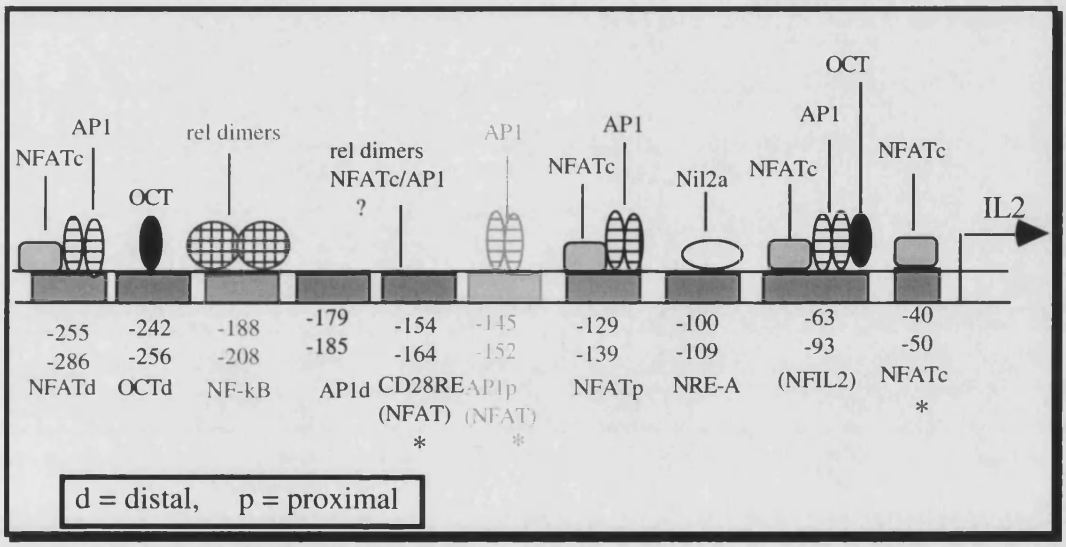

Figure 1.8: Schematic representation of the IL 2 promoter (not to scale) (Fujita et al., 1983; Granelli-Piperno and Nolan, 1991; Hughes and Pober, 1996).

Mutational studies have also shown that the importance of each transcription factor varies depending on the type of cell that is activated. In primary T cells NF-kB and AP1p binding sites are the most important elements. The proximal NFAT site was also important whereas the distal one did not play such a crucial role but was still important (Hughes and Pober, 1996). The results were different in Jurkat cells, were NFATd was the most important (Hughes and Pober, 1996).

Reports have also suggested the presence of a negative regulatory element (NRE) capable of binding a protein called NIL-2a at high levels during anergy (Williams et

al., 1991; Becker et al., 1995). Negative transcriptional regulation may therefore be taking place in anergic cells (Becker et al., 1995). Further supporting this the AP1p site is able to negatively regulate transcription after anergic stimulation (Kitagawa-Sakakida and Schwartz, 1996). Most possibly the AP1 complexes that bind DNA after these conditions contain some of the negatively regulatory jun / fos proteins as discussed later.

1.5.1: NF-kB

NF-kB is a ubiquitous transcription factor, involved in transcription of many genes that participate in acute phase responses, inflammation, cell growth and lymphocyte activation (reviewed by Baeuerle and Henkel, 1994). c-rel$^{-/-}$ and p50$^{-/-}$ mice suffer several defects on the immune system (Sha et al., 1995; Caamano et al., 1998; Doi et al., 1997). Interestingly mutating the NF-kB DNA binding site in IL-2 promoter did not have such severe effects suggesting that NF-kB has other functions as well (Shapiro et al., 1996). All NF-kB / rel proteins share an N-terminal rel homology domain, containing the DNA binding and oligomerisation regions together with the nuclear localisation signal (Baeuerle and Henkel, 1994). However, a common aspect of rel proteins is their sequestration in the cytoplasm as inactive complexes by ankyrin repeats that act by masking the nuclear localisation signal.(Baeuerle and Henkel, 1994; Arenzana-Seisdedos et al., 1995). Class I NF-kB proteins (p50 and p52), contain the rel domain but are made up as precursors (p105/NF-kB1 and p100/NF-kB2 respectively) that contain the appropriate ankyrin repeats. Class II NF-kB proteins (p65/RelA, RelB and c-rel), have related C terminal transactivation domains and are retained in the nucleus via inhibitor proteins that contain the ankyrin repeats (Thompson et al., 1995; Franzoso et al., 1992; Whiteside et al., 1997). After activation the ankyrin repeats are removed and NF-kB / rel proteins are allowed to enter the nucleus. Any combination of rel proteins can be formed, but in order to be active it must contain at least one of the class II proteins. This is because class I proteins solely consist of the rel domain, which lacks a transactivation

function. The most common and stronger dimmers that induce gene expression contain p50 and p65. During more prolonged NF-kB activation the role of p65 seems to be substituted by c-rel (Baeuerle and Henkel, 1994).

The signals needed for the release of NF-kB proteins from their inhibitory ankyrin repeats, seem to involve serine/threonine phosphorylation and subsequent ubiquitin dependent degradation of the inhibitors (Henkel et al., 1993; Chen et al., 1995; Baldi et al., 1996; Sun et al., 1996; Brockman et al., 1995; Palombella et al., 1994). Recent studies on the signalling of the cytokines IL-1 and TNF have identified a number of kinases that are able to interact and phosphorylate IkB proteins (DiDonato et al., 1997; Cao et al., 1996; Malinin et al., 1997; Karin and Delhase, 1998; Meyer et al., 1996). The same kinase have also been implicated downstream of CD28 (Herhaj and Sun, 1998). These signals are believed to initiate limited proteolysis of the class I precursors, and complete degradation of the IkB proteins while bound to their rel protein. Contradicting the general consensus, a recent report has suggested that IkBα, can be tyrosine phosphorylated and released without degradation from p50/p65 heterodimers allowing them to enter the nucleus and activate NF-kB dependant transcription (Imbert et al., 1996). This suggests that there is more than one way of activating NF-kB, allowing a complex but tight regulation of its activity.

Of the IkB inhibitors IkBα which contains 5 ankyrin repeats is the most studied (Brown et al., 1993; Chiao et al., 1994; Klement et al., 1996). Receptors or inducers (e.g. TNFα, PMA) that inactivate and degrade IkBα result in a transient NF-kB activation because amongst the genes activated by NF-kB is that of IkBα (autoregulation) (Chiao et al., 1994; Klement et al., 1996). IkBε is also found in T cells and has 6 ankyrin repeats but seems to be regulated similar to IkBα (Whiteside et al., 1997). IkBβ is inactivated together with IkBα by receptors that result in persistent NF-kB induction (e.g. IL-1, LPS) (Thompson et al., 1995). It therefore seems that the cellular distribution of the different types of IkBs together with the type of signal that activates the cells will determine whether a transient or persistent

regulation of NF-kB activity takes place (Thompson et al., 1995). Additionally NF-kB activity is positively regulated by a third protein called bcl-3 (Lenardo and Siebenlist, 1994; Wulczyn et al., 1992) which is thought to remove p50 homodimers, that bind DNA and act negatively in resting T cells (Bryan et al., 1994; Franzoso et al., 1992; Lenardo and Siebenlist, 1994). Furthermore bcl-3 has a transactivation domain and is thought to utilise it and positively regulate transcription by interacting with p52. Overall it seems that full NF-kB activation may require both IkB degradation and bcl-3 induction. In fact antigenic stimulations do upregulate bcl-3 (Lenardo and Siebenlist, 1994).

NF-kB proteins have been suggested to play an important role in the discrimination of established Th1 and Th2 cell types. Comparative studies, have shown that TCR stimulation can mediate IL-2 transcription in Th1, but not in Th2 cells. This is suggested to be caused by the inability of TCR signals to translocate p65 in the nucleus of Th2 cells (Lederer et al., 1996a; Lederer et al., 1994). Interestingly cAMP which is increased more in Th2 cells after TCR signalling, is known to decrease IL-2 production by decreasing NF-kB (Chen and Rothenberg, 1994). Additionally the lower increase of calcium after TCR signalling in Th2 cells (Sloan-Lancaster et al., 1997) may not help NF-kB activation. In respect to this the effect of PHA on NF-kB is blocked by inhibitors of receptor-operated calcium channels (Kanno and Siebenlist, 1996). Furthermore calcium dependent phosphatase calcineurin has also been shown to participate in the activation of NF-kB by aiding the modification of IkBα (Lai and Tan, 1994; Frantz et al., 1994; Steffan et al., 1995; Shatrov et al., 1997) and calcium dependent proteases are required to mediate the degradation of IkB proteins (Steffan et al., 1995). It is therefore clear that calcium is important for the correct activation of NF-kB.

The TCR and / or CD28 dependent signals inducing NF-kB in T cells are far from clear. Studies using naive T cells (Bryan et al., 1994) and jurkats (Lai and Tan, 1994) showed that whereas in a resting state, p50 is the only rel protein in the

nucleus, PMA alone can increase the levels of p50 and induce c-rel at low levels. When CD28 was also stimulated, this induction was seen at earlier time points, at higher levels and with a concomitant translocation of p65 in the nucleus. Overall PMA and CD28 could prolong NF-kB activation (Lai and Tan, 1994; Harhaj et al., 1996; Bryan et al., 1994). In another study the main role of CD28 was concentrated on c-rel translocation, allowing p50/p65 complexes to separate and bind with c-rel (Harhaj et al., 1996). The overall ability of CD28 to increase translocation of rel / NF-kB proteins in the nucleus have been attributed to rapamycin sensitive signals that prolong the degradation of IkBα (Lai and Tan, 1994). At the same time however it is possible that IkBβ which aids prolonged NF-kB activation (Harhaj et al., 1996), is also degraded. However, despite the clear ability of CD28 to induce NF-kB DNA binding activity, the effect on transcriptional activity is not as clear (Harhaj et al., 1996; Kanno and Siebenlist, 1996).

1.5.2: The CD28 response element (CD28RE).

The CD28RE was discovered via deletion studies in jurkat T cells, as an important element for IL-2 induction that required CD28 signalling for its engagement (Verweij et al., 1991), although later studies revealed that it can be transiently induced by prolonged PMA stimulations (Bryan et al., 1994; Lai and Tan, 1994). In fact Civil et al. have named this site NF-MAT due to its responsiveness to mitogens (Civil et al., 1996). Overall it has been suggested that the CD28RE together with the adjacent AP1 site on the IL-2 promoter are integration points of CD28 signalling (Shapiro et al., 1997; Butscher et al., 1998; McGuire and Iacobelli, 1997). Further research revealed that the complex mainly contains the NF-kB proteins c-rel, RelA and p50 (Verweij et al., 1991; Ghosh et al., 1993; Shapiro et al., 1997; Lai et al., 1995) suggesting that it is simply an NF-kB binding site. Another report however has found that the CD28RE (154-164) acts together with the adjacent AP1 / TRE site (146-155) and bind NF-kB together with AP1 proteins via the ability of rel proteins to interact with c-fos (McGuire and Iacobelli, 1997; Nolan, 1994; Shapiro

et al., 1996; Parra et al., 1998). An alternative report has also suggested that ATF1 / CREB2 proteins may also participate in the formation of the CD28 response complex (Butscher et al., 1998). Confusing the literature even more the CD28RE has also been found to bind NFAT in human resting T cells (Rooney et al., 1995; Godd et al., 1996). This is not very surprising due to the presence of a domain in NFAT that resembles the rel homology domain. Overall however, it seems that depending on the cell type and type of stimulation used the final composition of the CD28RC may differ. In light of the recent evidence concerning the ability of rel, AP1 and NFAT proteins to interact with each other the possible final complexes may be limitless (Shapiro et al., 1996).

1.5.3: AP1.

AP1 transcription factors are dimers consisting members of proteins of the fos family (c-fos, fosB, fosB2, fra1 and fra2), the jun family (c-jun, junB, junD and ATF2). DNA binding activity is not direct but is induced after a conformational change that takes place after dimerisation. Homodimers or heterodimers can be formed, but in order for the produced transcription factor to be active it must at least contain one of the jun proteins. Jun homodimers can be active but jun-fos heterodimers are much stronger in both their binding and transactivation ability. Since the IL-2 promoter only contains a weak (non-canonical) AP1 site, the presence of these heterodimers may be vital (Jain et al., 1995). Both c-fos and Fra1 make stable complexes with c-jun, but Fra1 lacks a transactivation domain and the dimmer is therefore inactive (Karin, 1995; Jain et al., 1995). Furthermore, not all jun family members act positively and junB can act negatively by competing with c-jun for interaction with the c-jun kinase JNK, which binds but does not phosphorylate junB (Karin, 1995; Jain et al., 1995).

Induction of c-jun and c-fos is one of the first nuclear events during T cell activation, followed be an induction of the IL-2Rα and IL-2 gene expression.

Induction of c-fos takes place with PMA, Ras and TCR stimulation of ERK. As a result, c-fos proteins heterodimerise with nuclear c-jun and bind DNA. Transactivation requires JNK, which can be partially activated by TCR signals, possibly by the induction of Rac-1 downstream of Ras (Genot et al., 1996). Further JNK activation requires CD28 signals (Jacinto et al., 1998; Coso et al., 1995; Kaga et al., 1998b; Su et al., 1994; Minden et al., 1995) as detailed in section 1.3.3.

1.5.4: NFAT

NFAT (Nuclear Factor of Activated T cells), is a composite transcriptional factor consisting of a cytosolic and an inducible nuclear component consisting of AP1 proteins (Boise et al., 1993a; Rao, 1997; Jain et al., 1993a; Northrop et al., 1993; Jain et al., 1992). Various cytosolic forms are known resulting from different genes or from splice variations of the same gene. They differ in cellular distribution and are thought to bind DNA with various affinities (Ho et al., 1995, Lyakh et al., 1997; Timmerman et al., 1997). All are however calcium dependent, require calcineurin for their translocation and are sensitive to CsA (Jain et al., 1993a; McCaffrey et al., 1993b; McCaffrey et al., 1993a; Flanagan et al., 1991). Mutant lines that can not sustain calcium do not translocate NFAT (Timmerman et al., 1996) and constitutive NFAT renders IL-2 induction independent of CsA.

NFAT1 or NFATp (p for pre-existing) is the main product of a gene that gives three isoforms via alternative splicing (NFAT1, NFAT3 and NFAT4) (Amasaki et al., 1998; Jain et al., 1995; Rao et al., 1997; Lyakh et al., 1997). Overall NFAT1 is characterised by an N-terminal proline rich region that may act as a transcriptional activator and an immediately adjacent 300 amino acid NFAT homology region (Masuda et al., 1997). The latter is vital for interactions with calcineurin and also contain the serine residues that are targeted by calcineurin (Nolan, 1994). The DNA binding motif of NFAT is distantly related to the rel homology domain, a fact that may explain the ability of NFAT proteins to interact with NF-kB DNA binding sites

in some cases (Nolan, 1994; Rao et al., 1997; Jain et al., 1995). The same domain is also responsible for dimerisation of NFAT proteins and interactions with AP1 proteins (Jain et al., 1995; Timmerman et al., 1997).

Calcineurin is required for the dephosphorylation of NFAT and the unmasking of nuclear translocation signals that aid translocation to the nucleus. It was until recently believed that calcineurin simply dephosphorylates NFAT, but studies with NFAT4 have shown that it binds at the N terminal region and enters the nucleus as a complex with NFAT4 (Shibasaki et al., 1996; Beals et al., 1997a). In the nucleus a competitive activity of kinases and the phosphatase, may determine the phosphorylating status of NFAT4 and its localisation (import or export) (Shibasaki et al., 1996; Scott et al., 1997). Thus, continuous calcineurin activity is probably required for NFAT4 nuclear localisation by competing with a kinase (Shibasaki et al., 1996). Consequently the strength of the calcium intensity and how sustained it is may be an important regulatory factor in NFAT activity. In fact a recent study clearly showed that NFAT activation requires a sustained, although not necessarily strong calcium rise (Colmetsh et al., 1997). The opposing role of kinases is clearly seen from the fact that PKC inhibitors stop the relocation of NFAT to the cytoplasm after ionomycin withdrawal or CsA (Scott et al., 1997; Timmerman et al., 1996). Thus in resting T cells a dynamic equilibrium must exist between calcineurin and kinase activities (Scott et al., 1997). Glycogen synthase kinase 3 (GSK3) and PKA are possible kinases regulating NFAT (Beals et al., 1997b; Klemm et al., 1997). GSK3 phosphorylates serine residues near other serines previously phosphorylated with PKA (e.g. ser 245), suggesting that the two kinases collaborate (Beals et al., 1997b). Furthermore, in T cells GSK3 is inhibited after TCR signalling (Beals et al., 1997b) while calcineurin is activated, thus favouring NFAT activation.

Calcineurin specifically aids nuclear translocation, but has no effect on the DNA binding ability or the ability to bind the nuclear components which are made up of AP1 proteins (jun and / or fos) (Jain et al., 1993b; Jain et al., 1993a;). AP1 proteins

seem to use their DNA binding regions to further attach NFAT1 with DNA, therefore aiding the assembly and stabilisation of the NFAT complex (Jain et al., 1993a). The signals that are responsible for the activation of AP1 proteins are therefore also required for NFAT activity (Jain et al., 1992; Jain et al., 1993b; Boise et al., 1993a; Rao, 1997). Thus, TCR signals via Ras, Rac and the MAPK pathway together with CD28 signals via Rac-1 and the JNK/SAPK pathway, are important for NFAT activity (Rao, 1997; Genot et al., 1996). CD28 signals have also been suggested to induce a CsA insensitive pathway that aids a partial NFAT translocation / activation and IL-2 induction when acting with PMA (Ghosh et al., 1996; Nebl et al., 1998; Lyakh et al., 1997). It is not clear what pathway is induced by CD28 in this case but an alternative phosphatase may be activated, like ceramide activated protein phosphatase (CAPP) (Dobrowski et al., 1993). In support to this IL-2 transcription has been shown to be inhibited by ocadaic acid, an inhibitor of this and other phosphatases (type 1, 2A, and 5) (Nebl et al., 1998). Alternatively since these studies were performed with CD28 antibodies which can induce calcium, a different calcium dependant but CsA independent phosphatase may be induced. In support of this, one study reported that a constitutive form of calcineurin is unable to fully substitute for ionomycin and induce NFAT together with PMA (Frantz et al., 1994).

NFAT1 is mainly found in resting T cells (Amasaki et al., 1998; Jain et al., 1995; Rao et al., 1997; Lyakh et al., 1997) and is responsible for most DNA binding activity of NFAT on the promoter of IL-2 and of other cytokines (Amasaki et al., 1998). However NFAT1 is not vital since NFAT1$^{-/-}$ cells can still produce most cytokines with the exception of IL-4, CD40-L and Fas-L which are decreased slightly (Xanthoudakis et al., 1996). Therefore, redundancy with other NFAT proteins must take place (Timmerman et al., 1997). The most possible candidate is NFAT2 or NFATc (c for cytosolic) which is found at lower levels in resting T cells, but mainly in activated T cells and especially in activated thymocytes (Jain et al., 1995; Rao et al., 1997; Amasaki et al., 1998). Recent studies have suggested that

NFAT proteins may play a crucial role in the differentiation of Th1 or Th2 cells. Transcription of the IL-4 gene for example is decreased in NFAT1$^{-/-}$ mice (Kiani et al., 1997; Xanthoudakis et al., 1996) and the Th1 type phenotype is promoted. In contrast, similar studies with NFAT2 have actually concluded the opposite and suggests that this type of NFAT transcription factor may promote Th2 cell differentiation (Ranger et al., 1998; Rincon and Flavell, 1997; Yoshida et al., 1998). These observations suggest that certain signal that allow one NFAT protein to dominate over another may be involved in Th cell differentiation.

1.6: AIMS.

It is clear from the preceding discussions, that both CD28 and CTLA-4 are important regulators of the immune responses but it is far from understood how these receptors mediate their functions. The signals initiated by CD28 have been examined in more detail, but most studies have utilised antibodies to trigger the CD28 receptor. Antibodies however do not represent the most physiological engagement of CD28 and as other reports have shown, result in different signals than the physiologic CD28 ligands do (Nunes et al., 1994; Nunes et al., 1993). Importantly cross-linked CD28 antibodies are suggested to elevate calcium in the cells (Ledbetter et al., 1992) and initiate a number of TCR dependent pathways (e.g. Ras, PLCγ1) (Nunes et al., 1994; Ohnishi et al., 1995). In contrast CD80 and CD86 are not suggested to mediate these pathways (Nunes et al., 1994; Ohnishi et al., 1995; Lu et al., 1995). The pathways that are initiated by CD80 and / or CD86 must represent a more accurate and physiologic picture, but are less well studied. To further examine the role of these signals, the effect on T cell proliferation and the promoter of the IL-2 gene was examined. Since not all the transcription factors

involved in the expression of the IL-2 are inducible, it was the aim of this work to concentrate on the ones that are activated during the stimulation of the T cells (i.e. NF-kB, AP1 and NFAT). Using electromobility gel shift assays (EMSAs), the ability of these transcription factors to bind DNA after CD28 engagement was assessed in freshly purified human T cells and jurkat T cells. Furthermore the transactivation potential of these transcription factors was examined by using luciferase reporter constructs with promoters that either contain multiple copies of the DNA binding elements of each transcription factor alone or corresponding to the whole IL-2 promoter. Since the TCR and the CD28 receptor are thought to synergise, it was one aim of these studies to examine and determine at which of the transcription factors such a synergy occurs.

As with CD28, the examination of the function of CTLA-4 has also been performed via the use of CTLA-4 antibodies. Despite their informative value, studies with CD28 and CTLA-4 antibodies are not able to account for the competition of these two receptors for their natural ligands, as it would normally happen during an immune response. It is therefore important to understand and examine the conditions that are able to favour engagement of one or the other receptor and thus promote or antagonise T cell activation. It was therefore the aim of the work presented here to understand how CD80 can also act as a negative regulator of T cell activation.

Thus the aims of these studies were

1. Examine the costimulatory potential of CD80.
2. Determine the contribution of CD80 in the proliferative responses of T cells and the production of IL-2.
3. Examine the activation of the IL-2 gene in detail and determine which of the transcription factors that participate in IL-2 transcription are mainly affected by CD80.

4. Establish the ability of CD80 to also act as a negative regulator of T cell activation and determine the conditions that may promote this and at the same time minimise the costimulatory potential.

CHAPTER 2

MATERIALS AND METHODS

2.1: MATERIALS AND EQUIPMENT.

2.1.1: General chemicals

Unless otherwise stated all chemicals utilised were purchased from Sigma Chemical Company Ltd. Buffers and solutions utilised are detailed in **appendix 1**. Tissue culture media contained 10% FCS (Sigma Chemical Company Ltd.) and were supplemented according to the protocols detailed in **appendix 2.** Supplements were obtained from Gibco BRL technologies. Other tissue culture related materials were purchased as specified.

2.1.2: Antibodies

Some of the antibodies used for the studies presented here were purified in our laboratory from hybridomas obtained from the American Type Culture Collection (ATCC) (Rockville, Maryland USA) and included CD2 (OKTII), CD3 (OKT3), CD25 (HB8784), CD69, and HLA-DR4 (L243). The anti-CD14 (UCHM1) was a kind gift from P. Beveley (Jenner Institute, Compton UK). The anti-CD19 antibody BU12 was a kind gift from I. MacLennan (University of Birmingham, UK). The anti-CD80 (BB1), the anti-CD28 antibody (9.3) and the anti-CTLA-4 antibody (11D4) were kind gifts from P. Linsley (Bristol-Myers Squibb, Seattle, USA) whereas Fab fragments of CD28 and CTLA-4 antibodies where a kind gift from C. June (U.S. Naval Medical Institute, Bethesda). CTLA-4Ig and human recombinant IL-2 were kind gifts from Glaxo whereas goat anti-mouse IgG and anti-mouse polyvalent (anti-IgM, IgG and IgA) FITC was purchased from Sigma Chemical Company Ltd. Monoclonal anti-human IL-2 and anti-human IL-2Rα antibodies were obtained from R&D systems.

2.1.3: General equipment

Tissue culture plastics were purchased from Farhenheit Laboratory Supplies Ltd and cell culture was performed using sterile techniques in a laminar flow hood (Class II). 5% CO^2 humidified incubators kept at 37°C were used, in order to incubate all cells and perform assays. Proliferation assays were determined by a β liquid scintillation TopCounter (Hewlet Packard).

Cells were counted using a B.S. 748 haemocytometer (Neubauer) and centrifuged in 30ml universals or 50 ml falcons at 1,500rpm (350g) for 5 minutes and at room temperature, in Beckman GPR centrifuge or a Beckman GS-15R centrifuge (rotor S4180). Cell extracts were centrifuged in 0.6 or 1.6ml eppendorfs at 13,000rpm (15,000g) in the Beckman GS-15R centrifuge (rotor F2402).

All cells for FACS analysis were centrifuged in 5ml polystyrene round bottom tubes at 1,500rpm (350g) for 5 minutes in the Beckman GPR centrifuge or the Beckman GS-15R centrifuge (rotor S4180). FACS analysis was performed on a Becton Dickinson FACStar Plus, using a 100mW, 488nm argon laser with light being channelled by an FL-1 filter (520nm±20) and FL-2 filter (580nm±20).

DNA and RNA were quantified using the Ultraspec II spectrophotometer (LKB Biochrom), whereas protein levels and ELISAs were determined via the use of a plate reader (Dynatech MR500). Gels for EMSAs were performed by gel apparatus obtained from Biorad. Luciferase activity was measured using the TD-20/20 luminometer (Turner Designs).

2.2: CELLS

2.2.1: Cell lines.

2.2.1.1: CHO transfected cells.

Chinese Hamster Ovary (CHO) cells untransfected or stably transfected with the CD80 receptor, HLA-DR4 antigen or both as previously described (Sansom et al., 1993; Markie et al., 1993) were maintained in Glutamine free DMEM medium supplemented as shown in appendix 2. Expression of transfected molecules was routinely examined using FACS analysis (see figure 3.1).

CHO cells were grown on a 2-3 day cycle and once at a confluent state the cells were passaged. Specifically the medium in the flasks was removed by aspiration and the adherent CHO cells were washed with phosphate buffered saline (PBS). Cells were then incubated with trypsin-EDTA (GibcoBRL technologies) (2mls in 75cm^2 tissue culture flasks or 3mls in 125cm^2 tissue culture flasks), for five minute at 37^{o}C. Gentle tapping of the flask completely dislodged the cells after which medium was added (4 times the volume of trypsin used) to inactivate the trypsin. One tenth of the cells were left in the flask and supplemented with fresh medium to grow again, while the rest were used appropriately. Prior to use CHO and CHO-CD80 cells were fixed with glutaraldehyde, in order to stop any further growth. This procedure leaves the cell intact however allowing the surface molecules to be utilised. That way any side effects of CHO cells were eliminated. The cells were washed twice with PBS counted and then resuspended for 2-3 minutes with 0.025% glutaraldehyde (1ml for every 5 million cells) at room temperature and by agitating every few seconds. Fixing was terminated by the addition of excess complete medium (10 times) and the cells were washed with medium, recounted and resuspended at the required concentration. CHO-DR4 and CHO-DR4/CD80 cells

were pulsed for four hours with the stated concentrations of SEA or SEB prior to fixing.

For long term storage, approximately $5x10^6$ CHO cells were frozen under liquid nitrogen at the time of subconfluence (middle of 3 day culture) by pelleting (1500rpm centrifugation for 5 minutes) and resuspending initially in complete medium containing 40% FCS and then slowly adding an equal volume of 20% DMSO (made in complete medium again). Before freezing in liquid nitrogen the cells were store at -80°C overnight. To re-emerge from liquid nitrogen, cells were quickly warmed to 37°C and washed once before culturing in $75cm^2$ flasks in the normal way.

2.2.1.2: Jurkat T cells (J6s).

Jurkat T cells (J6, ATCC) were maintained in RPMI-1640 medium supplemented as shown in appendix 2. When in a confluent state (usually every two days), cells were split 1 in 10 and supplemented with fresh medium. They were usually seeded at a concentration of $0.2x10^6$/ml and let to grow at a confluent state ($1\text{-}2x10^6$/ml) when they were split at a ratio of 1 to 10 again. Jurkat T cells were used at subconfluent state for the assays. As with CHO cells, J6s were also frozen for long term storage, via the same protocol.

2.2.1.3: Cytotoxic T cell leukaemic lines (CTLLs)

Murine CTLL cells were grown at complete RPMI-1640 medium supplemented as specified in appendix 2 and cultured in a $25cm^2$ tissue culture flask using a three day feeding cycle. Cells were seeded at a concentration of $0.02x10^6$/ml (total volume 10mls) and fed with 20 units/ml human recombinant IL-2 (kind gift from Glaxo). After three days incubation, with the flask standing upright and when the cell density increased approximately 10 fold, most cells were removed washed extensively and used in the IL-2 assays. The rest (about 10%) of them were left to grow and follow the above cycle. As with CHO cells, CTLLs were also frozen for

long term storage. Approximately 10^5 cells (whole flask) were pelleted by a 10 minute centrifugation at 1,100rpm and resuspended in 1ml of ice cold RPMI-1640 medium containing 20%FCS and 10%DMSO. Prior to liquid nitrogen storage, cells were incubated at -20^{0}C and -80^{0}C for 24 hours each. All centrifugations involving CTLLs were performed at 1,100rpm (200g) for 10 minutes.

2.2.2: Human T cell preparations

2.2.2.1: Generation of purified resting T cells.

Blood was obtained from healthy male and female human donors, collected in heparinised 50ml falcon tubes (0.1% heparin), diluted 1:1 with PBS and layered into a lymphoprep gradient (Nycomed, 1.077g/ml density). 25mls of diluted blood was gently layered in 15mls lymphoprep. After a thirty minutes centrifugation at 1500rpm and at room temperature the top layer containing the serum was removed and the interface / buoyant layer, containing the peripheral blood mononuclear cells (PBMCs), was collected. The bottom layer (red blood cells) was discarded. PBMCs were washed three times with RPMI 1640 medium and were then counted. Typically 1x10^6 PBMCs/ml of blood were obtained. PBMCs were then placed in a petri dish at a concentration of 5x10^6/ml, and incubated for 1 hour at 37^{o}C in complete RPMI-1640 medium. Adherent cells were that way removed and the rest were collected and washed twice with medium. They were then incubated with 1μg/ml (at a final volume of 500μl) anti-DR4 antibody (L243) to remove any class II positive cells, anti-CD14 antibody (UCHM1) to remove any non adherent monocytes and anti-CD19 (BU12) to remove B cells. After a one hour incubation at 4^{o}C the cells were washed resuspended in 500μl RPMI 1640 medium and 100μl sheep anti-mouse IgG dynal beads per 50mls of blood and incubated for another 1 hour at 4^{o}C. During this time the beads bound the primary antibodies and a magnet was then used to remove the selected cells. The remaining cells which comprised the resting T cell population were washed with medium and resuspended at an appropriate concentration to be used in the relevant assay. Typically 5x10^5 resting T

cells were obtained for every ml of blood collected. Cells were more than 95% CD2 and CD3 positive.

2.2.2.2: Preparation and maintenance of peripheral blood T cell blasts.

For the preparation of T cell blasts, PBMCs purified as mentioned above were stimulated with staphylococcal enterotoxin A (SEA) (10ng/ml) which binds HLA-DR molecules on the surface of antigen presenting cells, such as monocytes, macrophages and B cells, within the PBMC population. The same cells also provided the costimulatory molecules that synergised with SEA to activate T cells. The antigen responsive blasts were cultured in complete RPMI 1640 medium at a concentration of 2-4x10^6/ml for 7-9 days with medium added every 2-3 days. The cells were used at certain points during or at the end of their culture.

2.3: METHODS

2.3.1: Proliferation assays.

2.3.1.1: Stimulation of resting T cells or PBMCs.

Proliferation assays were performed in triplicate, on 96-well flat bottom plates at a volume of 200µl per well in RPMI 1640 medium. 5x10^4 T cells (100µl) were added in the wells containing 50µl medium or 2x10^4 fixed CHO control or transfected cells and 50µl of medium containing other stimuli such as PMA and / or ionomycin, or soluble CD3 (OKT3) antibody at quadruple the final concentration. CD3 cross-linking in culture was achieved with an equal concentration of anti-mouse IgG. Alternatively, wells were pre-coated with OKT3 antibody at the specified concentrations for 18 hours prior to the start of the assay. When the effect of

inhibitors was tested, T cells were incubated for thirty minutes prior to the start of the assay with the inhibitors at the concentrations stated in the figure legends. Blocking antibodies (or isotype-matched mouse Ig) were also added to the cells at the start of culture at concentrations indicated in the figure legends.

2.3.1.2: Stimulation of T cell blasts.

As above proliferation assays were performed in triplicate, on 96-well flat bottom plates at a volume of 200µl per well in RPMI 1640 medium. $5x10^4$ T cell blasts (100µl) were added in the wells containing 50µl medium or $2x10^4$ fixed CHO or CHO-CD80 cells and 50µl of medium containing other stimuli such as PMA and / or ionomycin, or soluble CD3 (OKT3) antibody at quadruple the final concentration and crosslinked with an equal concentration of anti-mouse IgG. When the effect of inhibitors was tested, T cell blasts were incubated for thirty minutes prior to the start of the assay with the inhibitors at twice the stated concentrations. For supernatant transfer experiments $5x10^4$ activated T cell blasts in 50µl medium were incubated with 150µl of the supernatants (i.e. final dilution of 1.25 times) or IL-2 (100 units/ml).

2.3.1.3: Culture condition and determination of the proliferative responses.

As also stated in the figure legends, the plates were incubated at 37°C for 72 hours (for T cells and PBMCs) or 24 hours (for activated T cells) at which time point 50µl were removed for IL-2 production as detailed below. 1µCi of tritiated ^{3}H-thymidine (Amersham International plc) was added to each well and after an additional 18 hours incubation the plates were harvested in to replica 96 well fibre filter plates, using a 96 well harvester (Filtermate 196 - Hewlet Packard). Radioactivity was measured via a β liquid scintillation counter (TopCount, Hewlet Packard). The counted CPM, represent the ^{3}H-Thymidine incorporated in the DNA of the cells during DNA synthesis and hence cell division. It is therefore an indirect measure of proliferation. Data are plotted as mean values of triplicate wells and represent at

least three independent experiments unless stated otherwise in the figure legends. Standard errors were always less than 10% of the mean value.

2.3.2: IL-2 cytokine detection.

2.3.2.1: CTLL assays

In order to examine the levels of IL-2 produced by the stimulated cells, the ability of supernatants obtained from these cells at 72 hours (see above at 2.3.1.3), to induce proliferation on the IL-2 dependent cell line CTLL was tested. Assays were performed in triplicate as the proliferations, but in 96-well round bottom plates. CTLLs were used at the third and last day of their culturing cycle (see above), and washed thoroughly to remove any presence of IL-2. The viability of the cells was checked by trypan blue, and was typically more than 90%. CTLLs were then resuspended at $1x10^5$/ml in RPMI 1640 medium (see appendix 2) and 50μl ($50x10^3$ cells) were added in each well. An extra 50μl were added which contained either dilutions of the samples taken from the proliferation assays, or known amounts of human recombinant IL-2 (Kind gift from Glaxo). IL-2 was used from 0 to 40 units/ml in 2 fold dilutions for a standard curve to be obtained (1 unit was equivalent to 1ng of IL-2). The assays were incubated for 18 hours at which point 0.5μCi of ^{3}H-thymidine was added for an additional 6 hours. The cells were then harvested and radioactivity was counted as with the proliferation assays. The standard curve obtained from the proliferative levels of CTLLs stimulated with known concentrations of IL-2 was used in order to estimate the levels of IL-2 present in the samples.

2.3.2.2: ELISA

IL-2 levels were also determined by human IL-2 ELISA DuoSet (Genzyme diagnostics). The protocol followed was the one supplied by the manufacturers. Briefly, 96 well MaxiSorp surface plates (NUNC) were initially coated with a capture antibody (monoclonal mouse anti-human IL-2) overnight at 4°C and the

samples obtained from the proliferation assays and standards were added on the wells for 1 hour at 37°C, after blocking for 2 hours with BSA. The secondary antibody (biotynalated polyclonal rabbit anti-human IL-2) was added for another hour at 37°C and the detection reagent (Horseradish peroxidase conjugated streptavidin) for 15 minutes. In between all these steps at least 3 thorough washings took place. At the end, TMB substrate solution (Sigma Chemical company Ltd) was added for 10 minutes and after the addition of an equal volume of stop solution (2M H_2SO_4) optical density was measured at 450nM (Dynatech MR500).

2.3.3: FACS analysis

Surface staining for CD2, CD3, CD25, CD28, CD69, CD80 and HLA-DR4 was performed by the addition of 50µl corresponding antibody (1µg/ml) on $2x10^5$ cells for 30 minutes at 4°C. Cells were then washed with PBS and any primary antibody bound by the cells was detected using anti-mouse polyvalent-FITC (Sigma) at 50µg/ml. As a control the staining of cells treated with only the secondary antibody was also determined. Cells were either stained directly or fixed with 1% paraformaldehyde and 1% FCS (in PBS) and analysed within 3 days.

For CTLA-4 staining, T cells ($2x10^5$) were left unstimulated or stimulated with either PMA (5ng/ml) alone or PMA(5ng/ml) + Ionomycin (1µM). In order to allow for receptor re-cycling and maximise staining, anti-CTLA-4 antibody (11D4, a gift from Dr P. Linsley) or control antibody (anti-CD14 UCHM1) were added at 1µg/ml to cultures at 37°C during the stimulation period (1 to 4 hours). Subsequently, the cells were fixed in 1% paraformaldehyde for 5 min, washed and resuspended in 50µl of PBS containing 0.1% saponin to permeabilise the cells. Primary antibodies were detected using anti-mouse polyvalent-FITC (Sigma) at 1/50 dilution in 0.1% saponin and cells were analysed by FACS. Traces shown are representative of at least three independent experiments.

For staining of dead cells, propidium iodide was added to the cells prior to FACS analysis, at a final concentration of 1μg/ml.

2.3.4: Electromobility gel shift assays (EMSA)

These assays were used to detect the binding of nuclear proteins to DNA corresponding to the transcription factor binding sites of the IL-2 promoter. It involved the stimulation of cells, the preparation and quantification of their nuclear extracts, the radioactive labelling of DNA and the running of the nuclear protein and DNA mixture in a gel.

2.3.4.1: Cell stimulations for nuclear extractions.

A minimum of 10 million T cells (resting or previously activated) were resuspended at $2x10^6$/ml and stimulated in a 12 well plate for 8 hours in RPMI 1640 medium. Equivalent stimulations were performed with 5 million jurkat T cells at a concentration of $1x10^6$/ml. Unless stated otherwise cells were stimulated with 5ng/ml PMA and 1μM ionomycin. CHO transfectants expressing CD80 were added at a ratio of 1:3 T cells. Inhibitors were incubated with the cells for thirty minutes prior to stimulation. After the incubation time was completed, 50μl aliquots from each stimulation was removed, placed in a 96-well flat bottom plate in triplicates. These samples were incubated for an extra 16 hours followed by a final 18 hour incubation with ^{3}H-Thymidine. This measured the proliferation rate of the T cells induced by each stimulation. As in other proliferation assays samples were also used for IL-2 measurements. The rest of the stimulated cells were used for the preparation of the nuclear extracts.

2.3.4.2: Nuclear extractions.

After the 8 hour incubation nuclear extractions were performed with a method (Andrews and Faller, 1991) derived from the large scale procedure of Dingham et al. (Dingham et al., 1983). Briefly the cells were washed with cold PBS and

resuspended in 1.6ml eppendorfs with 400µl of cold hypotonic buffer A (10mM Hepes, 1.5mM $MgCl_2$, 10mM KCl, 1mM DTT) at pH 7.8 supplemented with 0.2mM PMSF, 1ng/ml pepstatin and 1ng/ml leupeptin prior to use (see appendix 1 for details). After a 15 minute incubation on ice the lysed cells were vortexed for 10 seconds and then pelleted at 13,000rpm for 10 minutes. They were then resuspended in 50µl of buffer C (20mM Hepes pH 7.8, 1.5mM $MgCl_2$, 0.42M NaCl, 1mM DTT, 0.2mM EDTA and 25% glycerol) at pH 7.8 and supplemented with 0.2mM PMSF, 1ng/ml pepstatin, 1ng/ml leupeptin (see appendix 1 for details). During a 20 minute incubation on ice the nuclei were lysed by high salt extraction. Cellular debris was removed by centrifuging at 13,000rpm for 2 minutes and supernatants containing the nuclear proteins was collected. The extracts were quantified for protein as detailed below. They were stored at -80°C after flash freezing in liquid nitrogen.

2.3.4.3: Bio-Rad DC protein assay.

The Bio-Rad protein assay is based in the differential colour change of a dye in response to various protein concentrations, due to the binding of the reagent (Coomassie brilliant blue G-250) to basic and aromatic amino acids of proteins. Briefly 5µl of each sample is mixed with 25µl of a reagent A (supplemented with 20µl of solution S for every 1ml) and 200µl of reagent B. 5µl of known concentrations of BSA (0-3mg/ml) were used as standard. After a 15 minute incubation at room temperature optical density is measured at 750nm by a microplate reader (Dynatech MR500). Each nuclear extract was diluted to a final concentration of 0.75mg/ml in buffer c mentioned above.

2.3.4.4: Oligonucleotide labelling

Double stranded oligonucleotides corresponding to the NF-kB (Promega), AP1 (Promega), and NFAT (Santa Cruz Biotechnology Inc.) binding sites of the IL-2 promoter were radiolabelled with γ-^{32}P, transferred from radiolabelled γ-^{32}P ATP, via the action of the bacterial T4 polynucleotide kinase (Promega). The oligonucleotides used had the following sequences:

NF-kB	5' AGTTGAGGGGACTTTCCCAGG 3'
AP1	5' CGCTTGATGAGTCAGCCGGAA 3'
NFAT	5' GAGGAAAATTTG 3'

NF-kB and AP1 oligonucleotides were labelled by adding 2.5µl of DNA (1.75pmoles/µl), in a 25µl mixture containing 1XPNK buffer (Promega), 25µCi γ-^{32}P ATP (Amersham International plc), and 15 units of T4 PNK (Promega). For NFAT oligonucleotide 3.5µl were used (20ng/µl or 1.3pmoles/µl). The reaction mixture was incubated at 37°C for 10 minutes after which 1µl of 0.5M EDTA was added to inhibit the enzyme. After diluting to 50µl with TE buffer all oligonucleotides were stored at -20°C, for up to two weeks. Before use the incorporation of radiolabelled γ-^{32}P was measured by placing 1µl in Whatman paper and washing three times with sodium bisphosphate (pH7), once with MQ water and once with ethanol. These washes removed all the excess uncorperated label. The remaining activity was measured using a β liquid scintillation counter (Hewlet Packard TopCount). Oligonucleotides were then diluted appropriately at 40,000CPM/µl.

2.3.4.5: Electromobility Gel shifts Assay (EMSA)

Nuclear extracts were mixed with labelled oligonucleotides at the last step of the process. Typically 12µl of extract (corresponding to 9µg of protein) was added to 6µl binding mix containing 1µg poly dI-dC (Pharmacia Biotech), 7.5% glycerol, 38mM KCl and 0.6mM $MgCl_2$ (see appendix 1 for detail). The concentrations corresponded to the final volume of 20µl reached after the addition of 2µl oligonucleotide (80,000CPM), which took place after a 10 minute incubation. A further 30 minute incubation at room temperature, allowed the DNA to bind the protein.

The samples were then loaded in a 5% v/v native polyacrylamide gel made up with 0.5x TBE, 5% v/v acrylamide (NBL Gene Sciences Limited), 1% w/v APS

(Northumbria Biologicals, UK) and 0.09% v/v TEMED (BDH Chemicals). At the no extract control, containing just the oligonucleotide 2μl of 6% v/v loading dye (40% w/v sucrose and 0.25% bromophenyl blue in mili-Q water) was added. This was used as a marker of the extent the gel has run. After about 2 hours at a constant voltage of 150V, the gel was removed from one of the plates and fixed (with 10% v/v methanol, 10% v/v glacial acetic acid and 5% v/v glycerol) to avoid diffusion of the protein complexes in the gel. After transferring it on a filter paper and drying it for about one hour at 70°C, it was placed in a cassette with a film for autoradiography. Exposure of the film usually took 12-16 hours but this varied depending on strength and extent of the DNA-protein binding.

2.3.4.6: Competition assays.

In order to establish the specificity of the obtained bands competition experiments were also performed. The procedure is exactly as above (2.3.4.5) with the only exception that prior to adding the labelled oligonucleotide, excess (100X) unlabelled DNA was added. The idea of the experiment is that if the excess DNA is irrelevant to the radiolabelled DNA it will not affect the binding reaction. If it is the same though the binding reaction would be overtaken by the unlabelled DNA and no bands would be detected after autoradiography.

2.3.4.7: Supershift assays.

These experiments were performed in order to identify the proteins present in the complexes observed. An antibody (1μg/ml) for a specific protein is therefore added during an additional 20 minute incubation after the incubation of the protein with the binding mix (see 2.3.4.5). Theoretically this can result in the decrease or disappearance of the bands intensity, if the antibody binds the DNA binding region. If a different region of the protein is recognised by the antibody, the molecular mass of the complex would be expected to increase and the band would migrate slower, causing a supershift. If the protein is not part of the complex no effect will be seen.

2.3.5: Reporter construct assays.

EMSA assays detect binding of proteins at their DNA binding sites, but do not give any information about the activity of the transcription factor. For this purpose artificial promoters were used which have the binding sites of the transcription factors in them, fused to the gene of luciferase (Luc). Detection of luciferase activity of the gene is dependant on the binding and activation of the transcription factors on the artificial promoter. the luciferase constructs utilised here were kind gifts from Dr David Williams (Peptide Therapeutics, UK) (Williams et al., 1995). The IL-2-Luc construct contained the whole human IL-2 promoter (-326 to +47). The NF-kB-Luc, AP1-Luc and NFAT-Luc constructs contained three tandem copies of the hIL-2 NF-kB (-198 to -187), the hAP1 (-152 to -145) and the hNFAT (-286 to -255) site respectively.

The reporter constructs were allowed to enter the cells by the method of electroporation. The electric shock results to pores in the membrane of the cell from which DNA enters. The cells are then incubated with fresh medium for the membranes to reseal and the cells to grow and were then stimulated as stated and cytoplasmic extracts were made for the activity of luciferase to be detected.

2.3.5.1: Transfection conditions.

Jurkat cells at a subconfluent state were resuspended at a concentration of 10 million/ml in complete RPMI medium. A volume of 500µl, was electroporated with 10µg DNA at a voltage of 300V and a capacitance of 960µF. The resulting time constant was generally around 18-21ms.

2.3.5.2: Cell stimulations for luciferase assays

After transfection cells were left in medium for 24 hours and then stimulated for 18 hours in a 24 well plate. Typically $0.5x10^6$ cells were stimulated for 18 hours in 1ml of medium and supplemented with another ml of medium containing various

combinations of CHO-CD80 cells (at a ratio of 1:3 jurkat T cells), PMA (5ng/ml final concentration), ionomycin (1μM final concentration) or anti-CD3 antibody (10μg/ml final concentration and cross-linked with an equal concentration of anti-mouse IgG).

2.3.5.3: Preparation of cytoplasmic extracts and measurement of luciferase activity.

Cells stimulated as above were washed with PBS and resuspended in 1XReporter lysis buffer (Promega), at a concentration of $10x10^6$/ml and left at room temperature for 15 minutes with an occasional mix. After an extra 5 minute incubation on ice the cells were vortexed and underwent a single freeze (in liquid nitrogen) and thaw cycle. Supernatants were collected after a 2 minute centrifugation at 13,000rpm. The samples were stored at -80°C. By mixing 20μl of the sample with 100μl of luciferase assay reagent luminescence was measured for 10 seconds with the TD-20/20 Luminometer (Turner Designs).

2.3.6: Reverse transcribed polymerase chain reaction (RT-PCR) studies.

The studies and experimental procedures detailed below were performed in order to detect the presence of specific mRNA molecules in the cytoplasm of the cells after stimulations. For this work reagents, tips and pipettes were strictly used for RNA work. All solutions were made up with diethylpyrocarbonate (DEPC) treated water (1ml DEPC for every 1000mls of mili-Q water).

2.3.6.1: RNA extractions.

RNA extractions were based on that Chomczynski and Sacchi method (Chomczynski and Sacchi, 1987). Specifically, $10x10^6$ cells were stimulated at a concentration of $2x10^6$ cells/ml with various combinations of 5ng/ml PMA, 1μM ionomycin, CHO cells (at a ratio of 1:3 T cells) or CHO-CD80 cells (also at a ratio

of 1:3 T cells) as specified. T cells were stimulated for 4 hours in a 24 well plate at which time point total RNA was extracted. All cells were initially washed with PBS and then resuspended in 500µl of solution D (denaturing solution, see appendix 1) in large eppendorfs. This was followed by the sequential addition of 50µl of 2M sodium acetate pH4, 500µl water saturated phenol (Ampligene) and 100µl chloroform-isoamyl alcohol (49:1). Between each of these steps the eppendorfs were mixed by inversion and at the end vortexed for 10 seconds before cooling on ice for 15 minutes. After a 10 minute spin (13,000rpm) at a microcentrifuge, the top aqueous layer was transferred into a fresh tube and supplemented with an equal volume of chloroform-isoamyl alcohol (49:1). Eppendorfs were again shaken vigorously and then span for 5 minutes (13,000rpm). This extraction was repeated once more before the final aqueous layer was precipitated with an equal volume of isopropanol at -20°C overnight. RNA was then spun for 10 minutes (13,000rpm), redissolved in 150µl of solution D and reprecipitated with an equal volume of isopropanol as above. After a final spin the RNA pellet was washed twice with 70% ethanol and once with 100% ethanol and was then left to air dry for about 20 minutes with the eppendorf standing upside down.

2.3.6.2: RNA quantification.

RNA were quantified using the Ultraspec II spectrophotometer (LKB Biochrom) according to the instruments instructions. Briefly 5µl of the RNA sample were diluted 25 times to a final volume of 125µl and OD was measured at 260nm and 280nm and the ratio was calculated. Typically the latter was between 1.8 and 2, indicating low levels of impurities. Concentration of RNA was calculated according to the equation: A_{260} x 40 x dilution (i.e. 25) = mg/ml RNA

2.3.6.3: Reverse transcription.

The following 30µl reaction was set up containing approximately 400ng total RNA, 0.4 units SuperScript reverse transcriptase (Amersham Pharmacia), 1X appropriate buffer (Amersham Pharmacia), 0.01M DTT, 0.5mM dNTPs (Amersham

Pharmacia), 1.5μM oligo d(T) (Amersham Pharmacia), 1.5μl of RNAse inhibitor (Amersham Pharmacia) and DEPC water up to 30μl. The reaction mixture was incubated at 37°C for 1 hour and stopped with a 5 minutes incubation at 95°C. Samples were kept on ice when PCR reactions were performed immediately. Alternatively they were stored at -20°C, but denatured at 95°C (for 10 minutes) before use.

2.3.6.2: PCR primer design.

Primers were designed using Mac vector and manufactured by Gibco BRL life technologies. The sequences of the primers utilised in these studies (5' to 3') were:

Forward IL-2 primer	GCA TTG CAC TAA GTC TTG CAC TTG
Reverse IL-2 primer	GCA TCC TGG TGA GTT TGG GAT TC
	giving rise to a 155bp fragment of the IL-2 cDNA.
Forward IL-4 primer	CTC ACC TCC CAA CTG CTT CCC
Reverse IL-4 primer	GTG GAA CTG CTG TGC AGT CGC
	giving rise to a 290bp fragment of the IL-4 cDNA.
Forward IL-10 primer	CAG CCC CTT GAG AAA CCT TAT TG
Reverse IL-10 primer	AGT GTG TCA CCC TAT GGA AAC AGC
	giving rise to a 190bp fragment of the IL-10 cDNA.
Forward IL-13 primer	TGG TTT GGA GCA TCA ACC TGA C
Reverse IL-13 primer	CGG ACA TGC AAG CTG GAA AAC
	giving rise to a 162bp fragment of the IL-13 cDNA
	and a possible weaker fragment of 82bp.

2.3.6.4.: PCR conditions

PCR reactions were set up with 1µl of the produced cDNA together with 1.5mM $MgCl_2$, 0.2mM dNTPs, 0.1µM of each of the primers, 4µl of Taq buffer (Promega) and 1 unit of Taq enzyme (Promega) and water up to 40µl. Reactions were covered with mineral oil and performed with a Robocycler gradient 96 (Stratagene). After an initial preincubation for 5 minutes at 95°C the following cycle was performed 30 times

-1 minute at 95°C

-1 minute at 54°C

-1 minute at 72°C

A final extension incubation at 72°C was performed before chilling the reaction mixtures at 4°C.

2.3.6.4.: Agarose gel electrophoresis of PCR products.

PCR products were run in 2%w/v agarose (high strength NySieve 3:1-FMC Bioproducts) gels. The gels were made by adding 3g of agarose in 150mls of 1XTAE buffer and boiling in a microwave in order to dissolve. After cooling at approximately 50°C, ethidium bromide was added at a final concentration of 0.5µg/ml. to allow visualisation of the DNA bands under UV light (UV transilluminator-UVP). The gel was poured immediately after the addition of ethidium bromide and left at room temperature for about 30 minutes to set. The gel was run for 2 hours at 80-100V. As shown in the figures samples were run adjacent to DNA molecular weight markers obtained form ϕX174 DNA digested with *Hae* III (Promega) and giving rise to the following bands: 1353, 1078, 872, 603, 310, 271, 194, 118, 72.

CHAPTER 3

CD80 AS A COSTIMULATOR OF PROLIFERATION AND IL-2 PRODUCTION

3.1: INTRODUCTION

Extensive investigations have been carried out into the regulation of T cell activation and have identified CD28 as an important T cell receptor in this process. Although studies using monoclonal antibodies for the receptor have clearly shown the costimulatory potential of CD28, the aim of this work was to examine the ability of CD28 to costimulate via its natural ligand CD80. For this, the ability of CD80 to synergise with TCR signals and induce T cell activation was examined. Signals of the TCR pathway were induced by CD3 antibodies, superantigens (SEA and SEB) and pharmacological agents like PMA and ionomycin. The ability of each of them to synergise with CD80, was examined and compared via the use of in *vitro* proliferation assays. Further comparisons were performed by examining the effect of pharmacological inhibitors that are thought to block CD28 signalling. Specifically, wortmannin, which inhibits the activation of phosphoinositide-3-kinase (PI3K) (Truit et al., 1994; Yano et al., 1993; Ward et al., 1995), rapamycin, which blocks the activation of p70S6 kinase (Pai et al., 1994; Chung et al., 1992; Price et al., 1992) and chloroquine, which is suggested to prevent the activity of aSMase (Landewe et al., 1995; Edmead et al., 1996; Hedin and Thyberg, 1985) were utilised. Experiments were performed with freshly purified human resting T cells which are thought to require costimulation of TCR signals for their activation. However, the importance and requirement of the costimulatory signals was also examined on activated T cell blasts, in order to investigate any differences dictated by the activation state of the cells.

3.2: RESULTS

3.2.1: CD80 as a costimulator of purified human resting T cells

In the experiments performed in this thesis CD28 engagement was achieved by the use of CHO cells transfected with the cDNA for the CD80 receptor and representative levels of CD80 on these CHO cells are shown on **figure 3.1**. The costimulatory potential of these CHO-CD80 cells was initially examined by their ability to synergise with signals initiated by antibodies for the CD3ε chain that mimic TCR engagement. CD3 antibodies were used cross-linked, by coating on plastic overnight. The results in **figure 3.2,** show that CD3 alone, was unable to induce a proliferative response except partially at the higher concentrations. However, the addition of CHO-CD80 cells (but not control CHO cells) could further activate the T cells and even induce proliferation of T cells activated with lower anti-CD3 antibody. Thus, CD80 seems to provide necessary signals that allows the cells to respond better after TCR stimulation. Interestingly the ability of CD80 to costimulate T cell proliferation reached a plateau (with 10μg/ml anti-CD3) but was reduced at the highest concentration of anti-CD3 used.

Similar results were seen when the effect of superantigens was examined. SEA and SEB were utilised bound on DR4 molecules expressed on CHO cells at levels represented in **figure 3.1**. CHO-DR4 cells were therefore pulsed with various concentrations of SEA **(figure 3.3a)** or SEB **(figure 3.3b)** and their ability to induce proliferation was examined. As above, whereas only high concentrations were able to partially activate T cells, the additional presence of CD80 on the CHO-DR4 cells, reduced the amount of superantigen able to induce a response by 100-10,000 fold. Thus, T cells triggered with low antigenic stimuli that were otherwise ineffective are costimulated productively by CD80. These data agree with others, that CD28 may act to decrease sensitivity of T cells to antigen and therefore reduce the threshold of

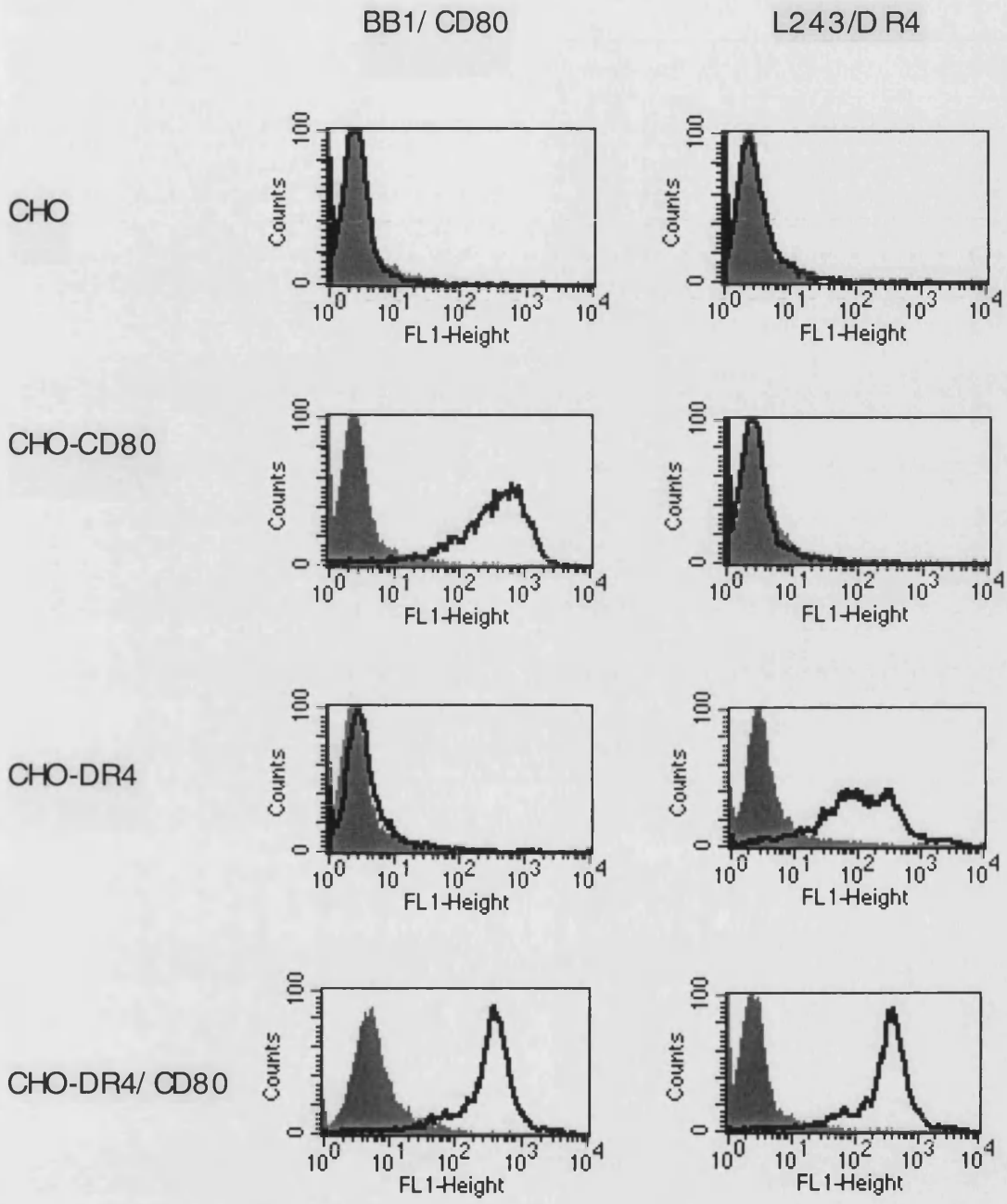

FIGURE 3.1: Expression levels of T cell ligands on CHO cells. Untransfected CHO cells and CHO cells transfected with cDNAs encoding CD80 and / or HLA-DR4 were stained for expression of these ligands as described in materials and methods. Closed (grey) histograms represent the background staining obtained with the secondary antibody alone, whereas the thick black line represents the positive staining. Cells were regularly stained and representative levels are shown.

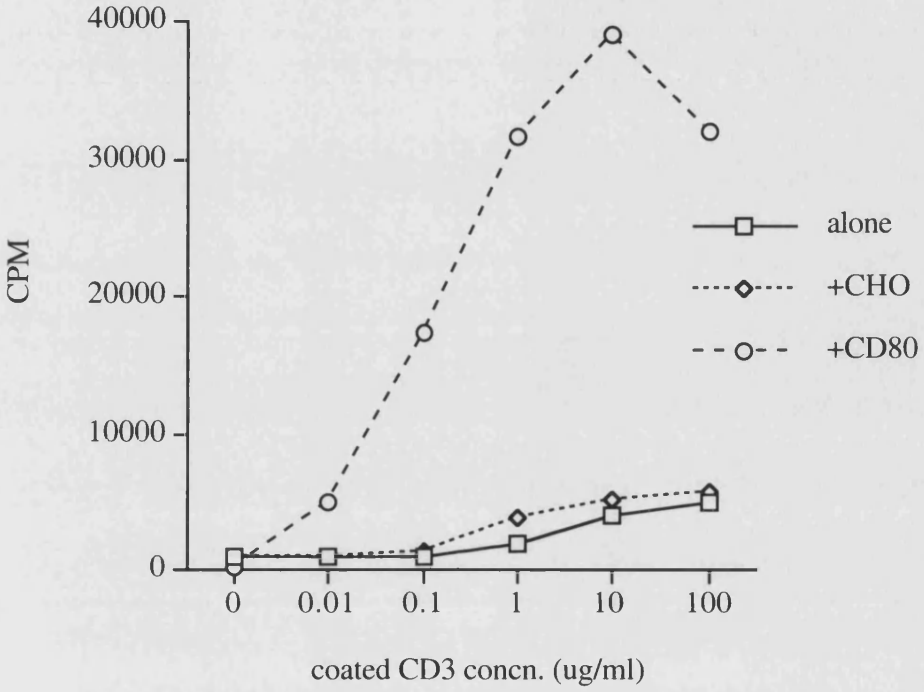

FIGURE 3.2: CD80 costimulates anti-CD3 antibody induced T cell activation. Purified human resting T cells were left untreated or incubated with different concentrations of anti-CD3 antibody (plate coated for 15-20 hours). The effect of the additional presence of CHO or CHO-CD80 cells (at a ratio of 1:3 T cells) was examined. Proliferation was measured at 72 hours by the incorporation of ^{3}H-thymidine during an additional 18 hour incubation.

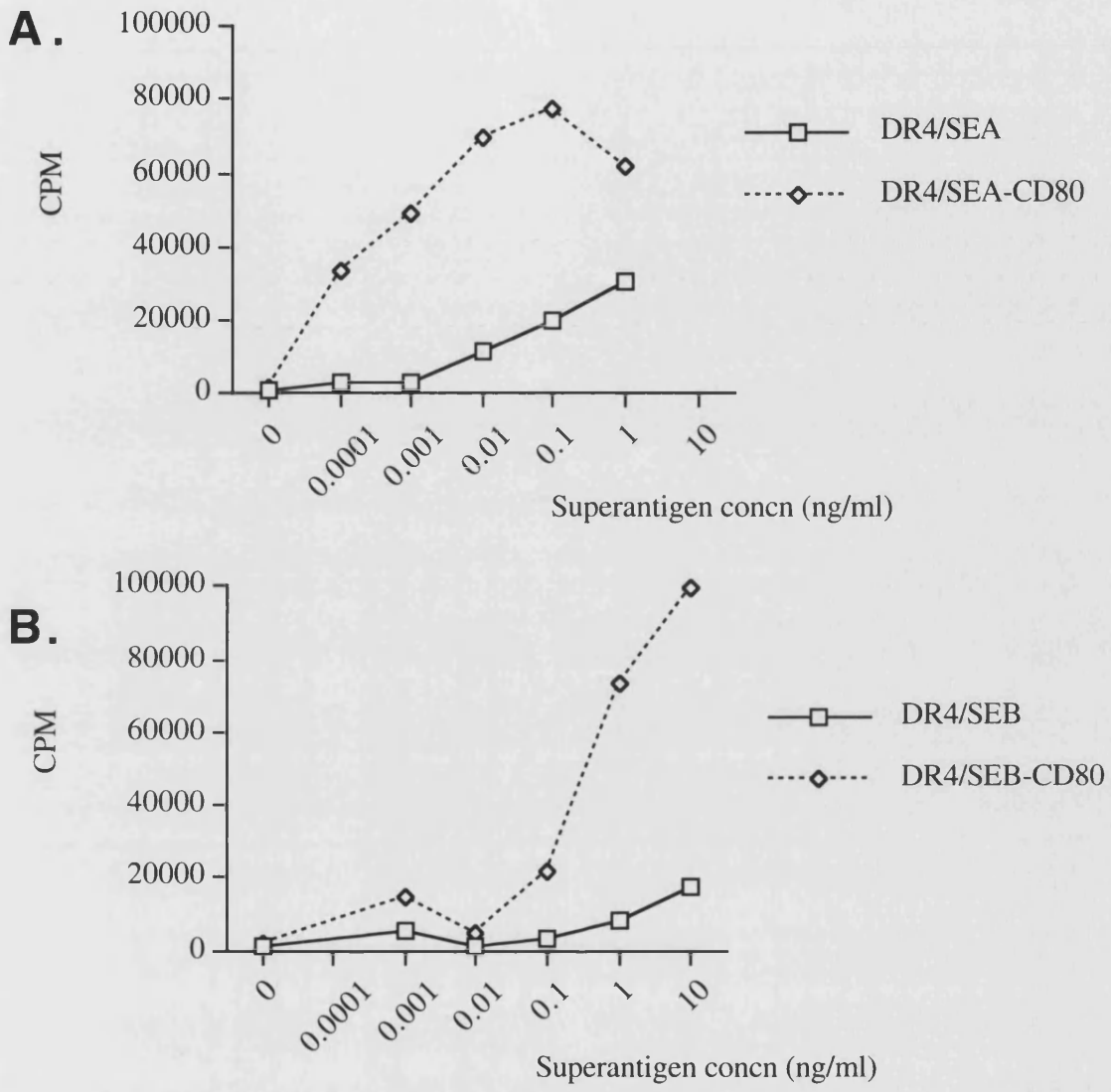

FIGURE 3.3: CD80 costimulates superantigen induced T cell activation. Purified human resting T cells were incubated with CHO-DR4 cells (at a ratio of 1:3 T cells) pulsed with different concentrations of superantigen SEA **(panel A)** or SEB **(panel B)** alone, or with equally treated cells also expressing CD80. Proliferation was measured at 72 hours by the incorporation of ^{3}H-thymidine during an additional 18 hour incubation.

TCR engagement required to initiate T cell activation (Wells et al., 1997; Thompson et al., 1993; Viola and Lanzavecchia, 1996). Additionally, like CD3+CD80 costimulation, DR4/SEA-CD80 responses reached a plateau and then decreased. Thus, strong TCR stimulations above an optimal level, seem to decrease the costimulatory ability of CD80. This is not seen with SEB, which is thought however to be less potent than SEA as a superantigen (Mahindate et al., 1995; Green et al., 1992) and as a result may not have reached optimal levels. This is clearly seen from the data presented here since, DR4-CD80 cells are able to activate T cells, when pulsed with lower SEA than SEB concentrations.

CD3 antibodies can be utilised in various ways. Cross-linking can take place by coating on plastic as shown above, but can also be performed using soluble CD3 via the aid of a soluble IgG cross-linker. Furthermore, soluble anti-CD3 can be used without cross-linking. These types of CD3 stimulation were therefore utilised at the optimal concentration of 10μg/ml, in order to examine whether they require costimulation via CD80 differentially. As the results show in **figure 3.4** CHO cells alone or CHO cells transfected with CD80 had no effect on the stimulation of T cells. However CHO-CD80 (but not CHO) cells, were able to synergise with all types of CD3 presentation and induce ^{3}H-thymidine uptake **(figure 3.4a)** and IL-2 production **(figure 3.4b).** Even soluble CD3 (without cross-linking) was able to synergise with CD80. This was rather surprising since this type of CD3 presentation is suggested to be unable to elevate calcium in the cells (Nakano et al., 1993; Sarkadi et al., 1991), an important downstream target of the TCR. In order to be sure that the antibody did not cross-link in the plastic during the culture period of the assay, wells were also pre-coated with FCS overnight. This did not affect the level of activation suggesting that all CD3 presentations tested can induce the necessary signals that will synergise with CD80 and costimulate T cells. Despite this, not all CD3 stimulations were identical. Specifically, cross-linking on plastic allowed some level of T cell activation even in the absence of CD80 costimulation although proliferation was accompanied by only limited levels of IL-2 **(figure 3.4b)**. Since

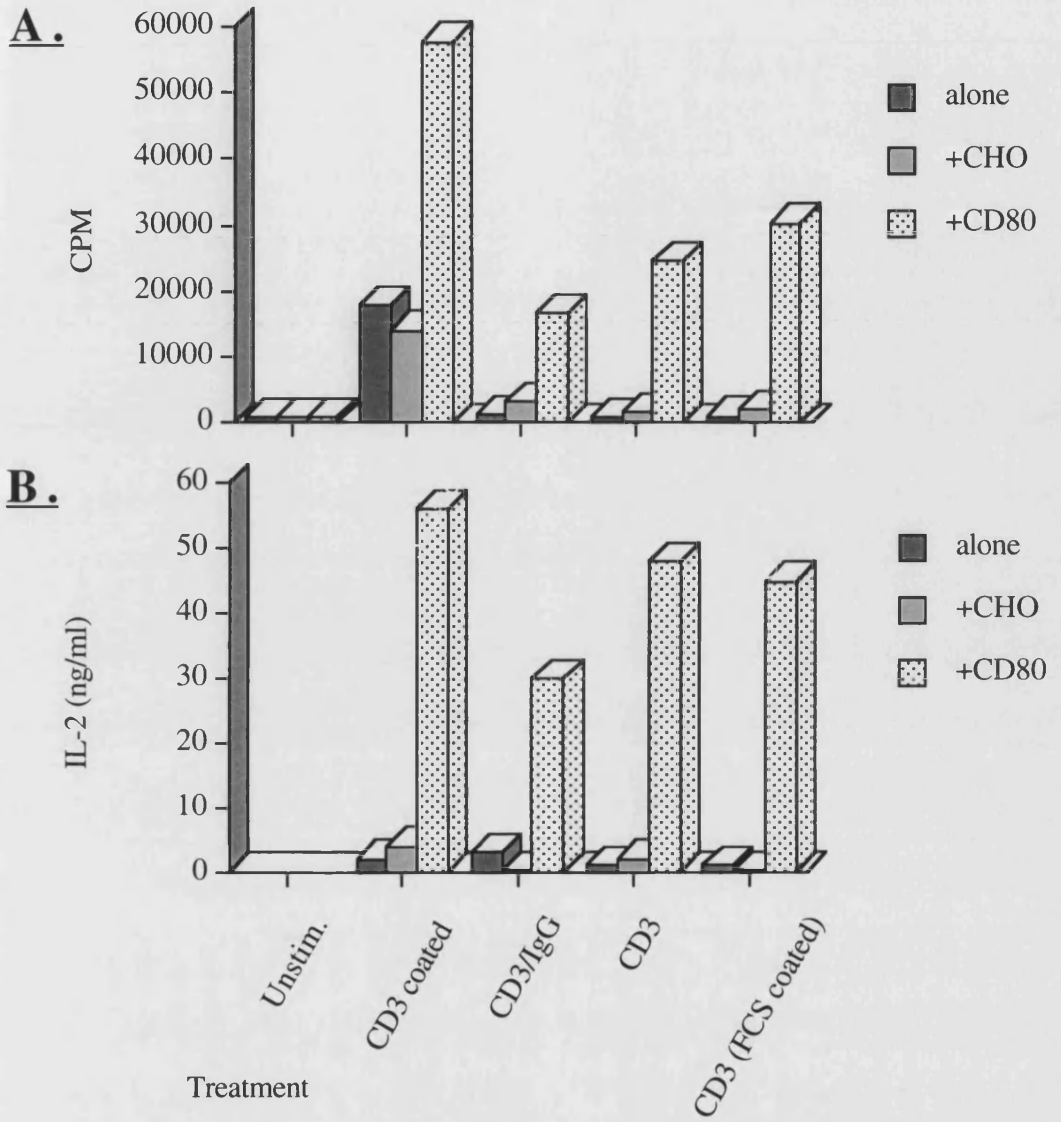

FIGURE 3.4: CD80 costimulates T cell activation induced by various presentations of anti-CD3 antibodies. Purified human resting T cells were left untreated or incubated with different presentations of 10μg/ml anti-CD3 antibody (cross-linked by plate coating for 15-20 hours-CD3 coated, cross-linked by soluble anti-mouse IgG-CD3/IgG, soluble-CD3 or soluble in wells pre-coated with FCS-CD3/FCS coated). The additional effect of CHO or CHO-CD80 cells (at a ratio of 1:3 T cells) was then determined. Proliferation **(panel A)** was measured at 72 hours by the incorporation of ^{3}H-thymidine during an additional 18 hour incubation. IL-2 **(panel B)** was determined by ELISA as detailed in material and methods.

proliferation after CD3 stimulation has been shown by others to require T:T cell interaction (Bentin et al., 1992) it is possible, that this type of CD3 presentation is able to upregulate certain molecules that are thought to interact with costimulatory receptors on the T cells themselves. Such possible receptors are SLAM (Aversa et al., 1997; Cocks et al., 1995) and CD6 (Osorio et al., 1998). Whereas the natural ligand of SLAM is unknown, the CD6 ligand (ALCAM) is present on activated T cells and could perform the above task (Osorio et al., 1998). However, if this is true the effect must be limited on the proliferative responses since in all cases CD80 was vital for the effective induction of IL-2 production. It is therefore clear from these results that CD80 directly or indirectly is utilised for the production of this cytokine.

CD28 has been shown to also costimulate T cell responses triggered by pharmacological agents like the phorbol ester PMA and the calcium ionophore ionomycin (June et al., 1989). PMA activates Ras and PKC (June et al., 1989; Takamara and Nakauchi, 1996; Downward et al., 1990), which are downstream targets of TCR signalling and represent the calcium independent pathways of the TCR. Ionomycin on the other hand increases calcium levels in the cell (June et al., 1989; Chatila et al., 1998). As **figure 3.5** shows PMA alone was unable to induce proliferation of T cells. However, when CD80 was also used a strong proliferative response resulted, which was not observed with control CHO cells. This response required a threshold of 0.5ng/ml PMA. However, to allow for fluctuations of responses, 5ng/ml was used in further experiments that examined CD80 costimulation under these conditions. Apart from synergising with CD80, PMA was able to synergise with ionomycin at even lower concentrations (0.05ng/ml) **(figure 3.5)**. These results suggest that compared to CD80, the calcium induced pathways that ionomycin initiates can costimulate PMA at lower concentrations. However, the results also show that calcium (as detected with the existing methods / techniques) is not an absolute requirement for T cell proliferation, since neither PMA nor CD80 are able to increase detectable levels of calcium in the cell (June et al., 1987; Thompson et al., 1993; Lu et al., 1995).

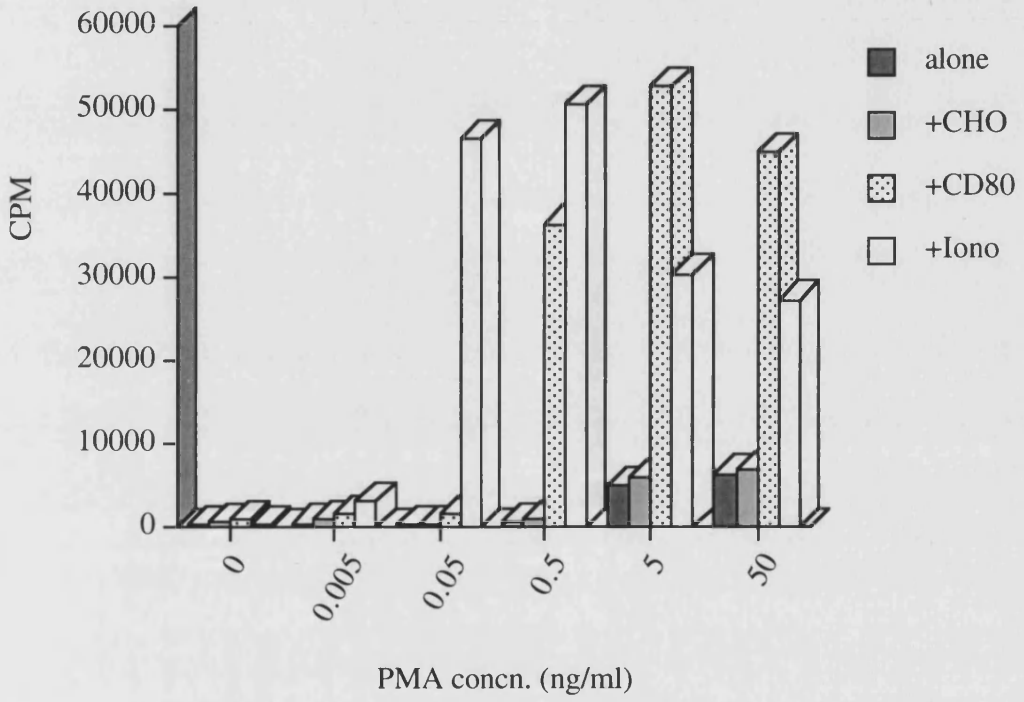

FIGURE 3.5: CD80 and ionomycin costimulate PMA induced T cell activation. Purified human resting T cells were left untreated or incubated with different concentration of PMA alone or in the presence of CHO cells, CHO-CD80 cells (both at a ratio of 1:3 T cells) or 1μM ionomycin. Proliferation was measured at 72 hours by the incorporation of ^{3}H-thymidine during an additional 18 hour incubation.

The synergistic action of PMA and ionomycin (P/I) was also seen, when IL-2 production levels were determined **(Figure 3.6)**. Whereas either stimulus was inefficient, their synergy resulted in IL-2 production. Since in the absence of CD80, TCR signals were found incapable of inducing IL-2 **(see figure 3.3a)**, this clearly suggests that pathways induced by P/I do not accurately represent TCR signals. Most possibly, PMA also activates some pathways of the CD28 signalling cascades. It is unlikely that ionomycin performs such a function since CD28 signals are suggested to be independent of calcium (June et al., 1987; Thompson et al., 1993, Lu et al., 1995). In contrast to the ability of ionomycin to synergise with PMA and induce IL-2, CD80 was unable to promote substantial levels of IL-2. This was surprising, since the proliferative responses induced by PMA+CD80 were equivalent to the ones induced by P/I **(figure 3.6).** However the results suggest that although calcium signals may not be absolutely required for proliferation, they may be vital for the production of IL-2. Additionally, it is clear from these data that the observed proliferation does not always correlate with the production of IL-2 and that other factors may also determine the final extent of the proliferative potential of a stimulation. Thus, IL-2 may not always be a vital and essential proliferative mediator of CD80 costimulation.

The ability of CD80 to act as a costimulator was finally examined on T cells stimulated with PMA and ionomycin together. Interestingly, the signals induced by low levels of PMA (0.005ng/ml) and 1μM ionomycin, which were unable to support proliferation, were effectively costimulated by CD80 **(figure 3.7)**. This suggests that low levels of PMA may mimic TCR signals more accurately. This costimulatory ability of CD80 disappeared as PMA concentration increased and its synergy with ionomycin became a more potent signal (0.05-0.5ng/ml PMA). In fact at high PMA concentrations (5-50ng/ml) the effect of CD80 (in the presence of ionomycin) turned into a negative one **(figure 3.7)**. As further discussed in chapter 5 this may represent the ability of CD80 to also act as a negative regulator of T cell activation via CTLA-4.

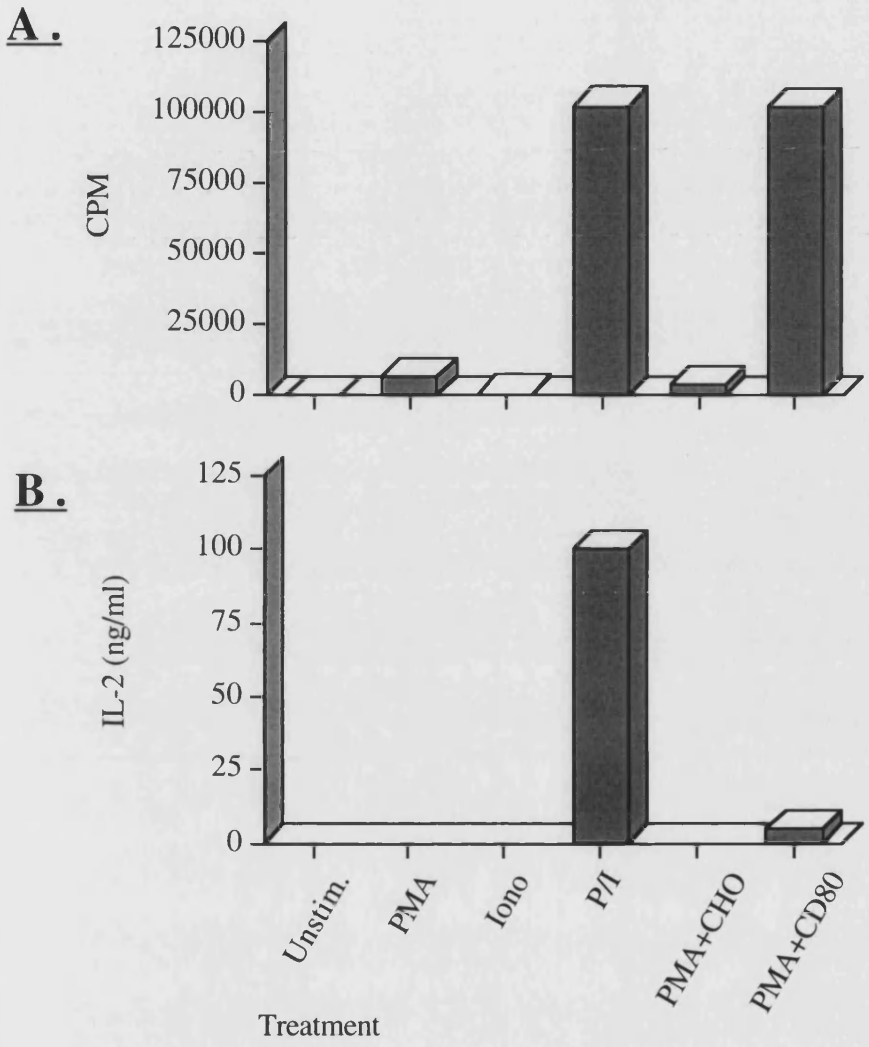

FIGURE 3.6: CD80 costimulates proliferation but not IL-2 production of PMA stimulated T cells. Purified human resting T cells were left untreated (Unstim.) or stimulated with 5ng/ml PMA alone (PMA), 1μM ionomycin alone (Iono), 5ng/ml PMA and 1μM ionomycin (P/I), 5ng/ml PMA and CHO cells (PMA+CHO) or 5ng/ml PMA and CHO-CD80 cells (PMA+CD80). CHO and CHO-CD80 cells were used at a ratio of 1:3 T cells. Proliferation (**panel A**) was measured at 72 hours by the incorporation of ^{3}H-thymidine during an additional 18 hour incubation. IL-2 levels (**panel B**) were determined by the CTLL assays as detailed in materials and methods.

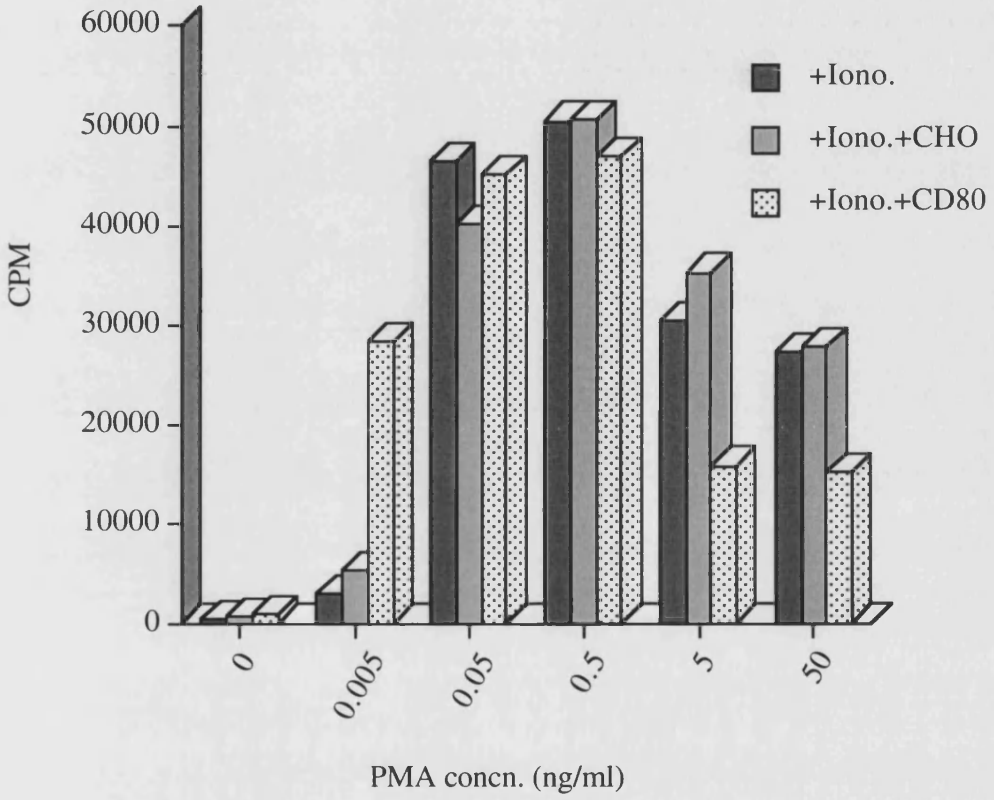

FIGURE 3.7: CD80 affects PMA and ionomycin responses differentially depending on the concentration of PMA used. Purified human resting T cells were left untreated or incubated with different concentration of PMA in the presence of 1μM ionomycin (+Iono.) alone or in the additional presence of either CHO cells (+Iono+CHO) or CHO-CD80 cells (+Iono+CD80) (both at a ratio of 1:3 T cells). Proliferation was measured at 72 hours by the incorporation of ^{3}H-thymidine during an additional 18 hour incubation.

3.2.2: Effect of pharmacological inhibitors on T cell activation.

The signals initiated by CD28 are suggested to include the activation of two lipid kinases called PI3K (Truitt et al., 1994; Prasad et al., 1994; Ward et al., 1995) and aSMase (Boucher et al., 1995; Edmead et al., 1996; Landewe et al., 1995). In order to examine the possible role of these pathways after CD28 ligation by CD80, inhibitors were used that are thought to prevent the activity of these enzymes. Wortmannin is thought to directly prevent the activation of the catalytic subunit of PI3K without affecting the recruitment of the enzyme on the cytoplasmic region of the CD28 receptor (Yano et al., 1993). Chloroquine is a less direct inhibitor which increases the pH of endosomes and lysosomes and as a result blocks the activity of acid hydrolases (such as aSMase) within these cell organelles, which need the low / acidic pH to perform their functions (Hedin and Thyberg, 1985). Finally the effect of rapamycin was also examined. This inhibitor is thought to prevent the activation of p70S6 kinase which is suggested to be a downstream target of PI3K (Chung et al., 1994; Pai et al., 1994; Hemmings, 1997). Specifically, rapamycin is suggested to prevent the activity of an enzyme called TOR (target of rapamycin), just upstream of p70S6 kinase (Chung et al., 1994; Kunz et al., 1993; Price et al., 1992).

The effect of these inhibitors was examined in human resting T cells stimulated with CD3+CD80 or PMA+CD80. As a control, the costimulation independent activation of T cells, induced by P/I was utilised. As **figure 3.8** shows, T cell activation induced by CD3+CD80 was blocked by wortmannin in a dose dependant manner. In contrast PMA+CD80 stimulation was only marginally decreased by wortmannin. In fact in some experiments no inhibition was observed. Finally P/I was unaffected by Wortmannin. These results clearly show that the signals that promote proliferation and result after the costimulatory activity of CD80, differ depending on whether CD3 or PMA is used as the primary signal. In support to this activation of PI3K has not been considered vital for the costimulatory activity of CD28 in some studies (Truit et al., 1995; Collette et al., 1997; Crooks et al., 1995). In the experiments

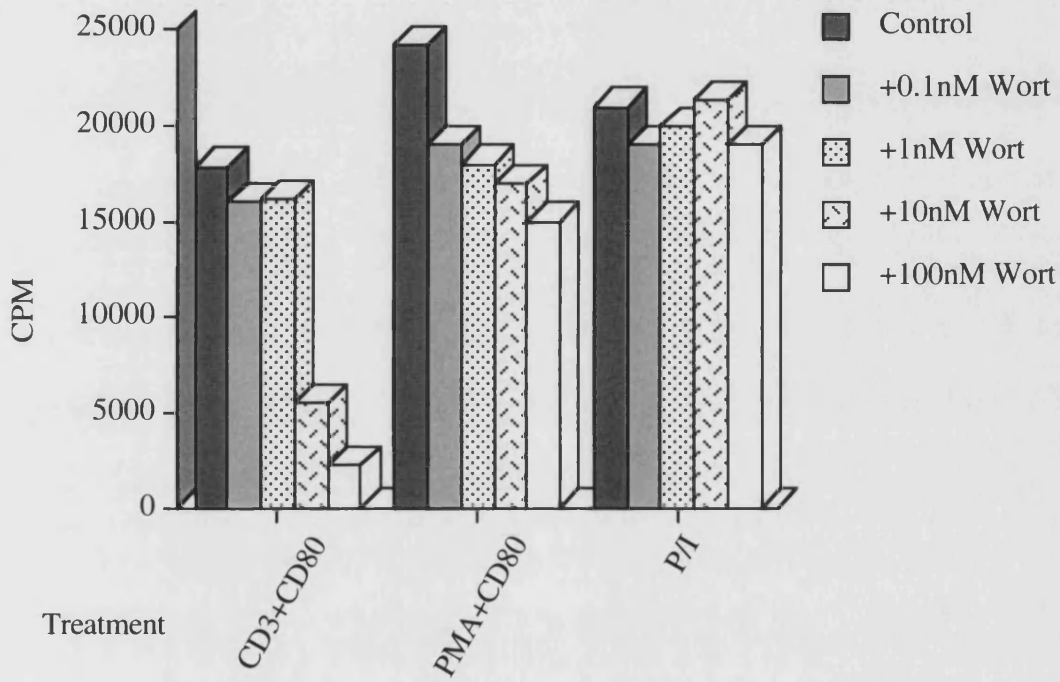

FIGURE 3.8: Wortmannin blocks CD80 costimulation of anti-CD3 antibody but not PMA induced T cell activation. Purified human resting T cells were left untreated (Control) or pre-treated for 30 minutes with different concentration of wortmannin (Wort) and stimulated with 10μg/ml soluble anti-CD3 antibody (cross-linked with anti-mouse IgG) and CHO-CD80 cells (CD3+CD80), 5ng/ml PMA and CHO-CD80 cells (PMA+CD80) or 5ng/ml PMA and 1μM ionomycin (P/I). CHO-CD80 cells were used at a ratio of 1:3 T cells. Proliferation was measured at 72 hours by the incorporation of ^{3}H-thymidine during an additional 18 hour incubation.

performed here, the PI3K pathway is suggested to play a crucial role when the TCR signals are induced by receptor activation (via CD3 antibodies), but not when activation is performed via PMA and / or ionomycin. One possible explanation for this result may be that PMA (but not anti-CD3 antibodies) is able to induce certain CD28 targets, further downstream of PI3K. Alternatively, PI3K may not be utilised under these circumstances. In fact PMA has been suggested to uncouple CD28 and PI3K interaction induced by CD80, although this is mainly seen at high PMA concentrations (Parry et al., 1996; Hutchcroft et al., 1995). Additionally, it must be noted that PI3K participates in the signalling of various receptors (Rudd et al., 1994; Ward et al., 1996; Ueda et al., 1995). Relevant to the experiments done here, PI3K is utilised by the TCR (Ueda et al., 1995; Rudd et al., 1994) and the IL-2 receptor (Taniguchi and Minami, 1993; Remillard et al., 1991; Shibuya et al., 1992). Although the activation of PI3K by CD3 is considered short-lived and of low magnitude, it may be important for the CD28 induced PI3K activation to be more effective after CD80 ligation. The effect of wortmannin would therefore not be restricted on PI3K induced by CD28, but also by TCR. Whereas this would affect signalling via CD3+CD80, the use of the post-receptor activator PMA may bypass this step, make PI3K less important and therefore contribute to the wortmannin resistance of PMA+CD80 stimulation. Finally the utilisation of PI3K during IL-2 receptor signalling (Taniguchi and Minami, 1993; Remillard et al., 1991) may also be relevant to the differential effects of wortmannin observed here. In contrast to CD3+CD80, IL-2 does not seem to be strongly induced after stimulation with PMA+CD80, which suggests that IL-2 signalling and thus PI3K may be less important. The combination of some or all these factors, may contribute to the ability of CD28 to signal independently of PI3K after PMA+CD80 stimulation.

Further downstream of PI3K, p70S6 kinase is thought to be activated and play an important role for cell cycle progression (Ming et al., 1994; Maurice et al., 1993). In many studies using other receptors (e.g. PDGF receptor) the activity of this enzyme is blocked by wortmannin, suggesting that it is a downstream target of PI3K (Franke

et al., 1995; Burgering and Coffer, 1995). In order to examine the importance of this enzyme on the signals generated after CD28 engagement, rapamycin was used. The results in **figure 3.9** show that like wortmannin, rapamycin prevented proliferative responses induced by CD3+CD80. In contrast stimulation with PMA+CD80 and P/I was only slightly affected by rapamycin. Thus, stimulation of T cells with PMA+CD80 may result in alternative signals that promote cell cycle progression without utilising the target of rapamycin (TOR) and / or p70S6 kinase. The may be explained by the ability of PMA to initiate CD28 signals further downstream than the site of action of rapamycin (TOR) (Pai et al., 1994; Ming et al., 1994), but others have actually shown the ability of rapamycin to prevent at least some of the signals induced by PMA and anti-CD28 antibodies (Lai and Tan, 1994). However, it is interesting that rapamycin is like wortmannin an inhibitor of IL-2 signalling (Kirken et al., 1997; Miyazaki et al., 1995). Thus, as suggested for wortmannin above, the resistance of PMA+CD80 proliferation to rapamycin may result from a lower requirement for IL-2 signalling under these conditions.

A second signal that is thought to be induced by CD28 is the activation of the aSMase pathway. In the proliferation assays performed **(figure 3.10)**, chloroquine was clearly found to prevent the ability of CD80 to promote T cell activation when CD3 or PMA was used as a primary signal. However the costimulation independent activation of T cells via P/I was also prevented. Although this may indicate that aSMase is universally important for T cell activation, the specificity of the action of chloroquine has been questioned. Whereas the chloroquine concentrations used here are not thought to be toxic and cause death, the broad action of chloroquine may affect the activity of other acidic hydrolases in the lysosomes (Landewe et al., 1995; Hedin and Thyberg, 1985). Additionally the effect of this drug on endosomes may affect CD28 indirectly, by interfering with its possible signalling capabilities during its receptor mediated endocytosis (Cefai et al., 1998). Thus, the effect of chloroquine in these experiments can only be interpreted with caution about its specificity as an inhibitor. To try and further examine the importance of aSMase in

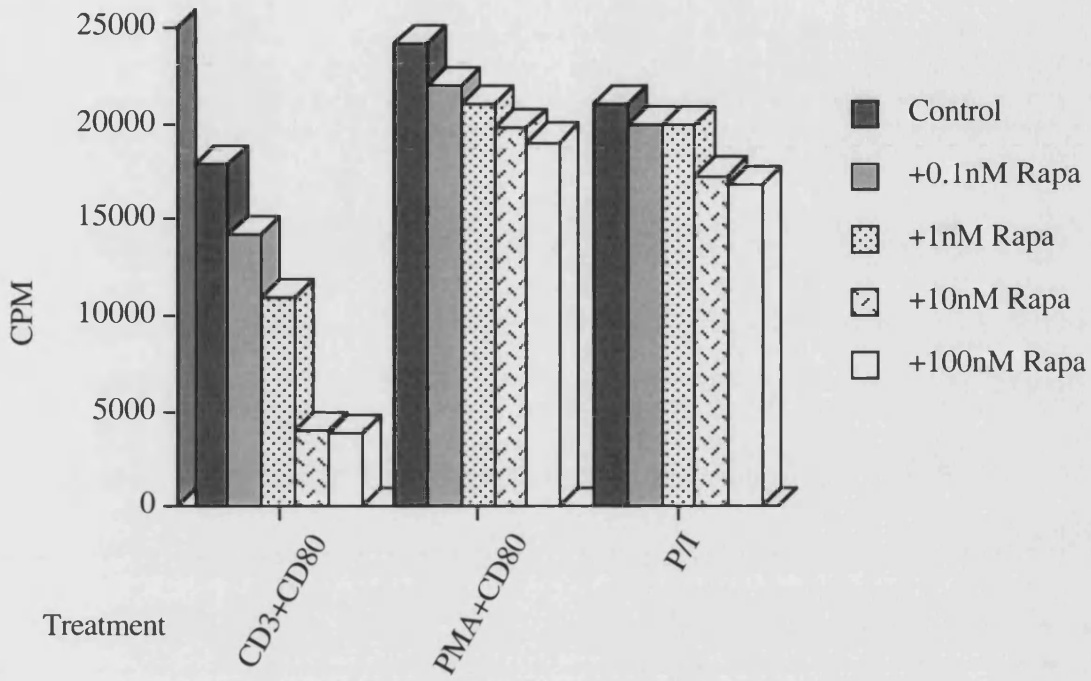

FIGURE 3.9: Rapamycin blocks CD80 costimulation of anti-CD3 antibody but not PMA induced T cell activation. Purified human resting T cells were left untreated (Control) or pre-treated for 30 minutes with different concentration of rapamycin (Rapa) and stimulated with 10μg/ml soluble anti-CD3 antibody (cross-linked with anti-mouse IgG) and CHO-CD80 cells (CD3+CD80), 5ng/ml PMA and CHO-CD80 cells (PMA+CD80) or 5ng/ml PMA and 1μM ionomycin (P/I). CHO-CD80 cells were used at a ratio of 1:3 T cells. Proliferation was measured at 72 hours by the incorporation of ^{3}H-thymidine during an additional 18 hour incubation.

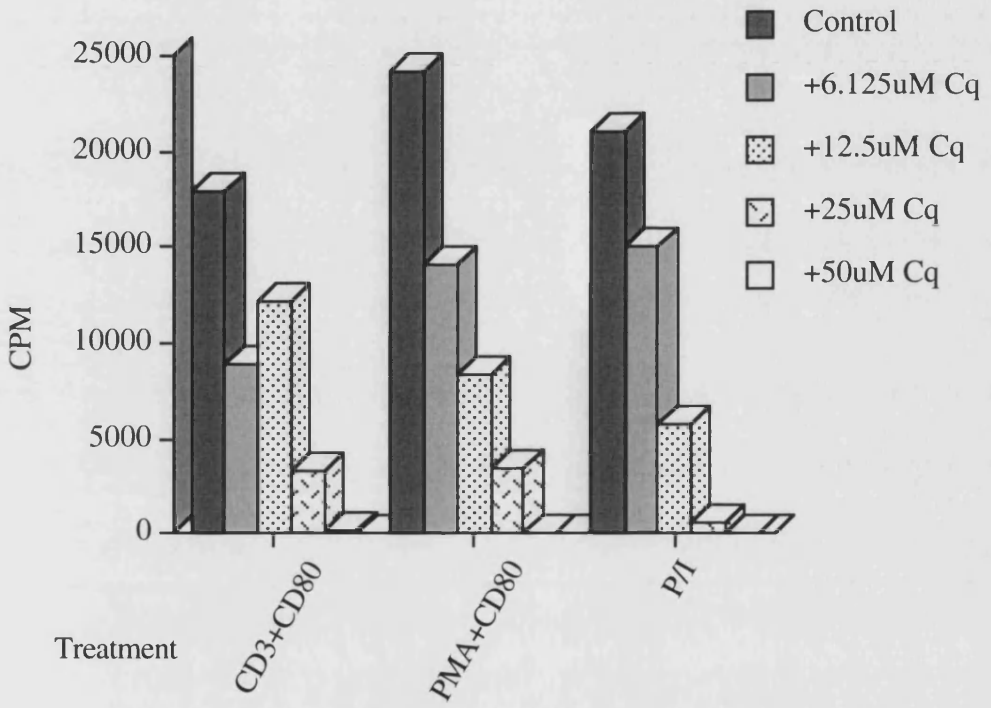

FIGURE 3.10: Chloroquine is a potent inhibitor of T cell activation. Purified human resting T cells were left untreated (Control) or pre-treated for 30 minutes with different concentration of chloroquine (Cq) and stimulated with 10μg/ml soluble anti-CD3 antibody (cross-linked with anti-mouse IgG) and CHO-CD80 cells (CD3+CD80), 5ng/ml PMA and CHO-CD80 cells (PMA+CD80) or 5ng/ml PMA and 1μM ionomycin (P/I). CHO and CHO-CD80 cells were used at a ratio of 1:3 T cells. Proliferation was measured at 72 hours by the incorporation of ^{3}H-thymidine during an additional 18 hour incubation.

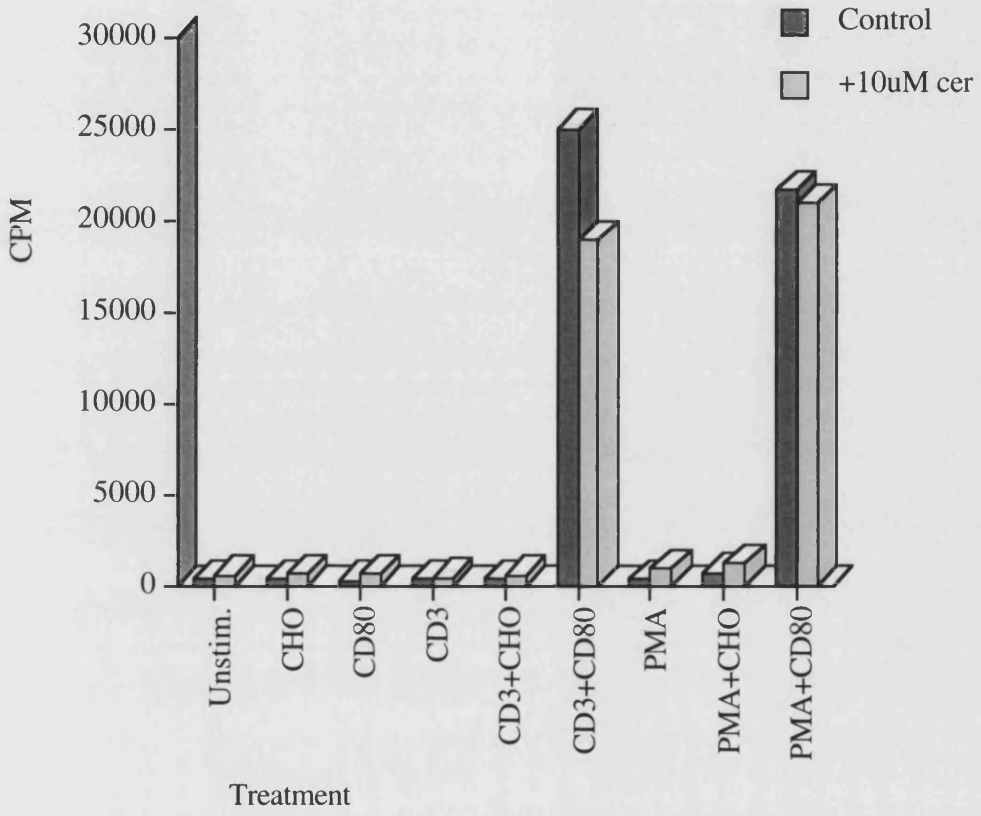

FIGURE 3.11: Ceramide is unable to substitute or enhance CD80 costimulation of either anti-CD3 antibody or PMA induced T cell activation. Purified human resting T cells were left untreated (Control) or treated with 10μM ceramide (cer). They were then left alone (unstim.) or stimulated with, 10μg/ml soluble anti-CD3 antibody (cross-linked with anti-mouse IgG) or 5ng/ml PMA together with CHO or CHO-CD80 cells (both at a ratio of 1:3 T cells). Proliferation was measured at 72 hours by the incorporation of ^{3}H-thymidine during an additional 18 hour incubation. The results are representative of two independent experiments.

T cell costimulation its product ceramide was utilised. Ceramide is a very versatile second messenger that can induce effects as diverse as apoptosis and proliferation (Hannun, 1994; Pushkareva et al., 1995; Hannun, 1996; Obeid et al., 1993). In T cells, 50μM ceramide induces apoptotic pathways (Jarvis et al., 1994; Obeid et al., 1993). In order to examine the effect of ceramide at a lower non-apoptotic concentration the ability of 10μM ceramide to costimulate T cells was examined. However, as the results show ceramide was unable to costimulate T cell responses initiated by CD3 or PMA and was therefore unable to substitute CD80 **(figure 3.11).** These data suggest that ceramide and therefore aSMase are not a main costimulatory signal downstream of CD28. Despite this, it is possible that the activity of aSMase is utilised and is important, but is not sufficient alone. To examine this possibility the ability of ceramide to potentiate CD28 signals was also investigated, but again no additional effect was observed on CD3+CD80 or PMA+CD80 stimulation **(figure 3.11)**. These results are inconclusive however for two reasons. Firstly, even if ceramide is important, the signals generated by CD80 in these stimulations may be strong enough not to require additional ceramide. Thus, to better examine the role of ceramide and therefore aSMase in T cell activation it will be better to examine the costimulatory potential of ceramide on T cells activated by CD3 or PMA and sub-optimal CD28 signals. Secondly, other metabolites of aSMase activity (e.g. sphingosine) (Merrill et al., 1996; Hannun, 1996; Guvillier et al., 1996; Kolesnick and Golde, 1994) may also be important in the costimulatory process. Generally more experiments are needed to examine the role of aSMase and confirm its role in costimulation as others have suggested (Boucher et al., 1995).

The experiments performed here clearly suggest that wortmannin, rapamycin and chloroquine can prevent the activation of T cells after stimulation with CD3+CD80. Since this signal also induces significant amounts of IL-2 the effect of these inhibitors was further examined in the context of IL-2 production. As **figure 3.12** shows all of them were able to prevent the induction of this cytokine. With caution concerning the effect mediated by chloroquine, these data suggest that the PI3K

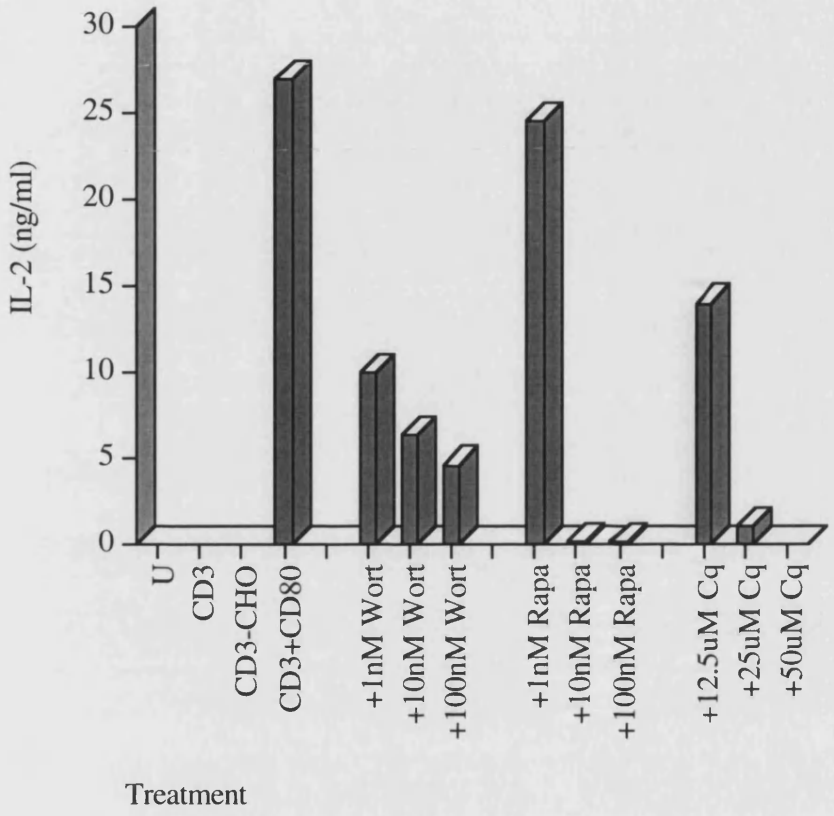

FIGURE 3.12: Wortmannin, rapamycin and chloroquine can prevent CD3+CD80 induction of IL-2. Purified human resting T cells were pre-treated for 30 minutes with different concentration of wortmannin (Wort), rapamycin (Rapa) or chloroquine (Cq) and stimulated with 10μg/ml soluble anti-CD3 antibody (cross-linked with anti-mouse IgG) and CHO-CD80 cells (at a ratio of 1:3 T cells) (CD3+CD80). IL-2 production was determined by the CTLL assay as detailed in materials and methods.

pathway and the aSMase pathway initiated by CD28 may be responsible for promoting the expression of IL-2. As a consequence, the effect of these inhibitors on the proliferation observed above may be due to their ability to inhibit IL-2 production.

3.2.3: CD80 as a costimulator of previously activated T cells

A number of studies have suggested that CD80 and / or CD86 act as costimulatory ligands via CD28 not only when T cell activation is initiated but also at later stages (Edmead et al., 1996; Damle et al., 1992) by possibly playing a role in maintaining T cell responses. To examine this possibility T cell blasts were prepared by the addition of superantigen SEA to a freshly purified PBMC population. The cells were cultured for 7 to 9 days and during this time used for subsequent experiments. Cells collected at the middle of the culture period (day 3 to 5), were used as activated T cells. Cells taken at the end of the culture period (days 7-9) represented a population of previously activated but more quiescent T cells. The actual specific days the cells were used varied from experiment to experiment, due to donor variability to stimulation and culture conditions. **Figure 3.13** shows the expression of certain receptors in the surface of T cell blasts at various stages of activation. As these results show, a number of PBMCs just before stimulation, lacked CD2, CD3 and CD28 expression. This was expected, due to the presence of non T cells in the population (i.e. monocytes, B cells and other APCs). However, the levels of these receptors quickly increased after activation. This results from the propagation of T cells after stimulation and the simultaneous death of the antigen presenting cells. By the end of the culture (day 9) most cells expressed CD2, CD3 and CD28. The other two surface molecules (CD25 and CD69) did not appear in the cells until after stimulation. CD69 rose first in the surface and its levels peaked by day 2 after which they started to fall back to resting levels. CD25 on the other hand reached peak levels at later time points (between day 4 and 6) but again returned to unstimulated levels by day 9.

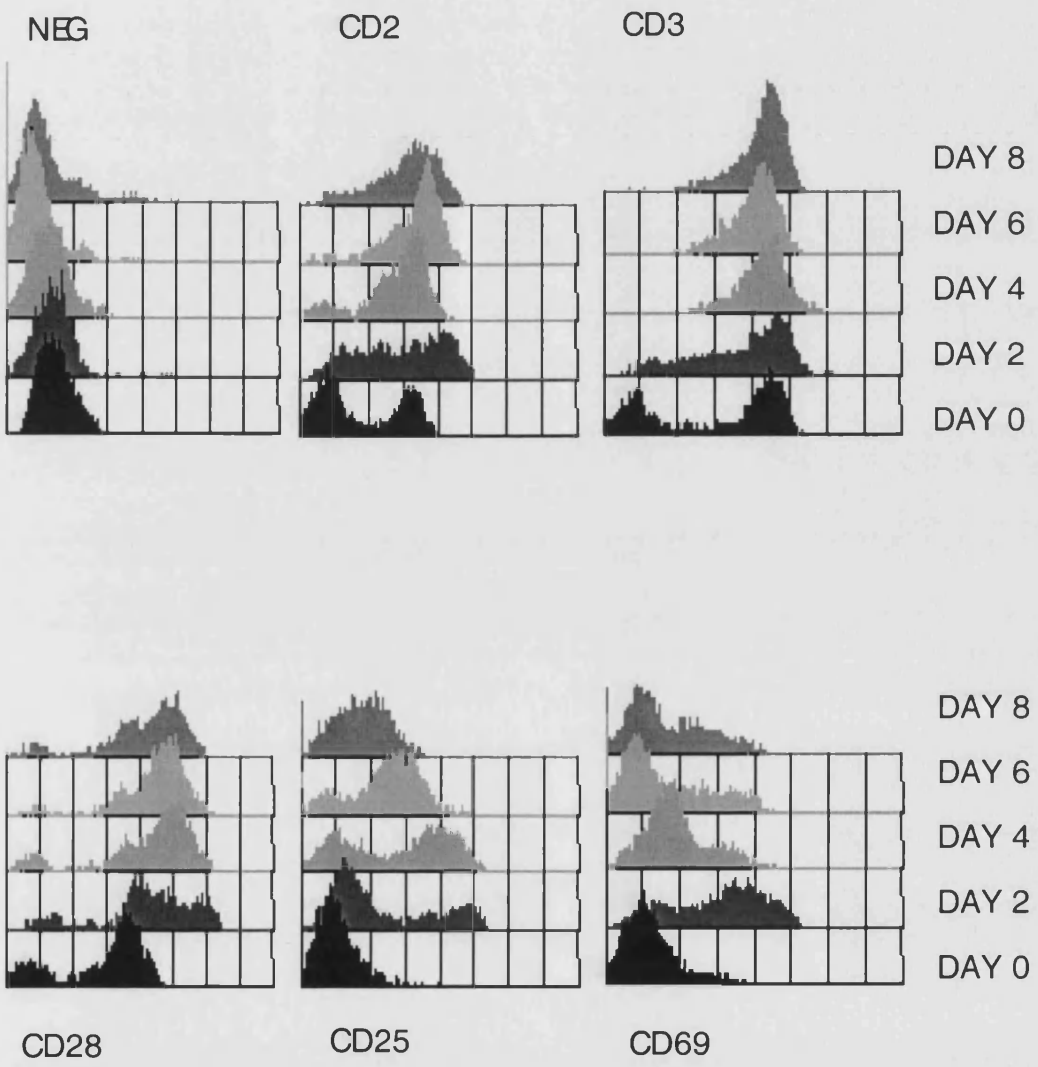

FIGURE 3.13: Surface marker changes in SEA activated PBMCs. SEA (10ng/ml) activated T cells were obtained at various days after activation and were stained for the surface expression of CD2, CD3, CD28, CD25 and CD69 as described in materials and methods. Control staining (NEG) represents the background levels of staining induced by the secondary antibody alone.

The ability of CD28 to induce or sustain proliferation of activated T cells, was initially investigated. Cells were extensively washed before stimulation in order to eliminate the presence of any proliferative factors from the original culture and therefore decrease the basal, unstimulated levels of proliferation. The effect of CD80 was compared to the one induced by each of the other T cell stimuli mentioned above. The results clearly show that in contrast to rested T cells, activated T cells responded to CD80 alone and resulted in a proliferative response above the basal levels observed with the untreated blasts at the same day **(figure 3.14a).** A similar effect can also be achieved by either PMA, CD3 or DR4/SEA alone. In contrast, at later stages when the cells reached a resting state these signals alone were not sufficient. The cells however were still capable of activation since they could be restimulated by the use of two signals **(figure 3.14b)**. These results suggest that activated T cells are less stringent in the signals that govern their proliferation. Interestingly, previous studies in our laboratory has suggested that this ability of CD80 to further activate T cell blasts is not accompanied by IL-2 production (Edmead et al., 1996). This, suggests that similar to the signals initiated by PMA+CD80, CD80 alone may be acting by prolonging cell division and sustaining proliferation of active cells via a mechanism that does not involve IL-2.

The differences between activated and resting T cells in their stimulation requirements prompted an investigation in to the effect of the inhibitors on these cells. Wortmannin, rapamycin and chloroquine were examined on activated T cell blasts. Interestingly stimulation of T cell blasts with CD80 was only weakly affected by wortmannin **(Figure 3.15)** but largely prevent by rapamycin. Although this may be a dose effect the ability of both inhibitors to act similarly in other cases (i.e. resting T cells), suggests that PI3K and p70S6 kinase are not always in the same signalling cascade. Chloroquine was the only of the three inhibitors that totally blocked T cell activation by CD80.

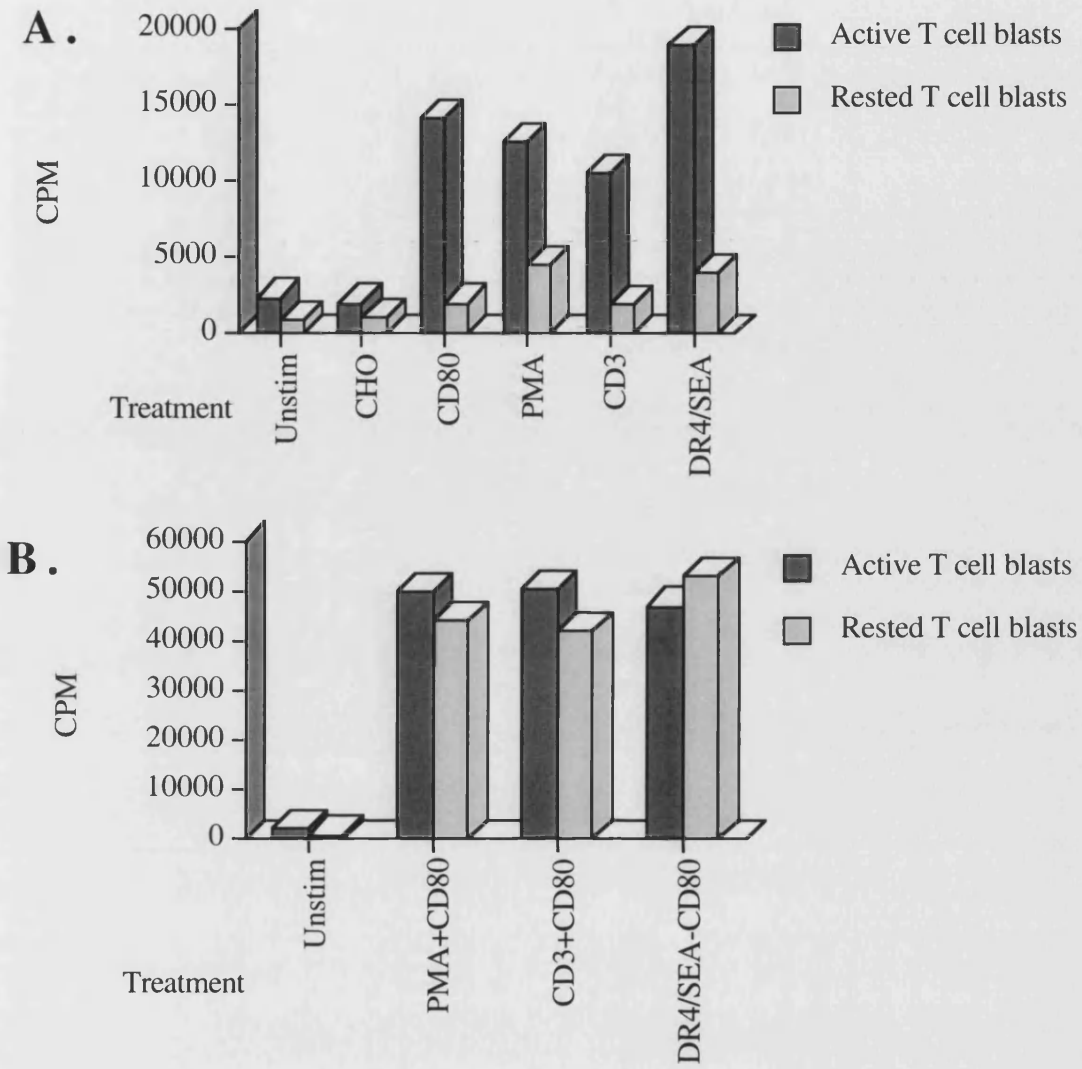

FIGURE 3.14: Unlike resting T cells, activated T cells can respond to one signal alone. SEA (10ng/ml) activated T cells were obtained at day 4 (active T cell blasts) or day 9 (rested T cell blasts) and were extensively washed. Cells were then stimulated with one signal **(Panel A)** or two signals **(Panel B)** as specified. CHO, CHO-CD80, CHO-DR4 and CHO-DR4/CD80 cells were used at a ratio of 1:3 T cells. Anti-CD3 antibody was used soluble (cross-linked with anti-mouse IgG) at 10μg/ml and PMA was used at 5ng/ml. Proliferation was measured at 24 hours by the incorporation of ^{3}H-thymidine during an additional 18 hour incubation. The results are representative of two independent experiments.

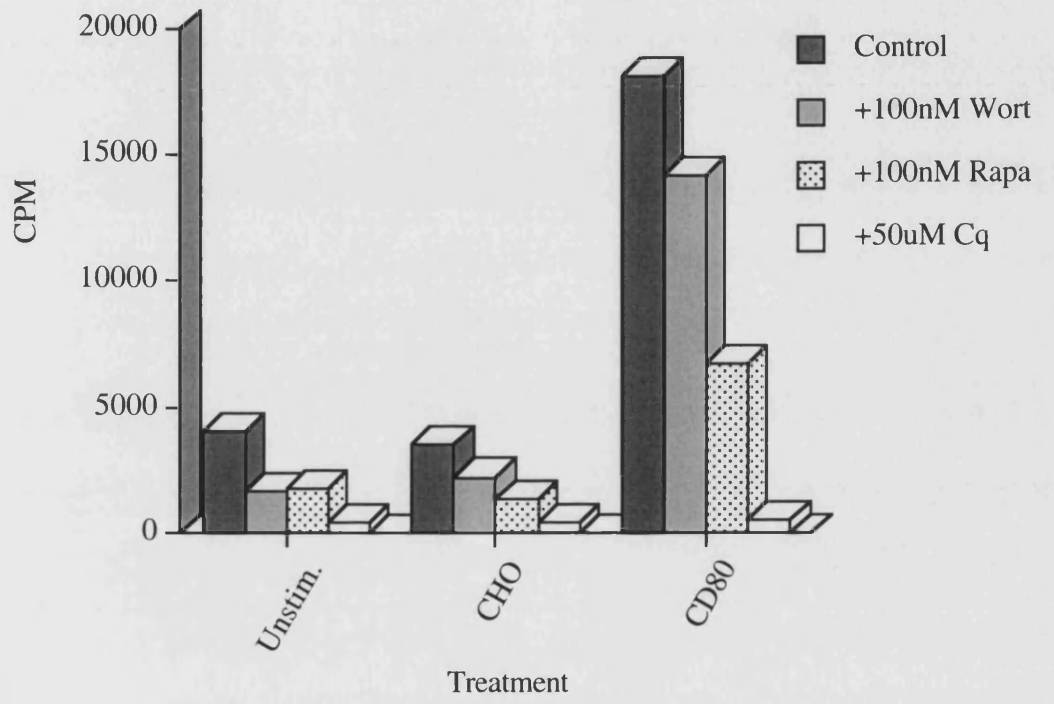

FIGURE 3.15: Effect of pharmacological inhibitors on CD80 costimulation of activated T cells. Day 4 SEA (10ng/ml) activated T cells were extensively washed and pre-treated for 30 minutes with the indicated concentrations of wortmannin (Wort), rapamycin (Rapa) or chloroquine (Cq). Cells were then left alone (Control) or stimulated with CHO or CHO-CD80 cells (at a ratio of 1:3 T cells) and proliferation was measured at 24 hours by the incorporation of ^{3}H-thymidine during an additional 18 hour incubation. The results are representative of two independent experiments

3.3: DISCUSSION

In the experiments performed during the whole of this thesis, CD80 transfected and expressed on the surface of CHO cells, was used as a ligand for CD28. The results presented in this chapter were therefore obtained from experiments designed to test the ability of these CHO-CD80 cells to costimulate T cell responses induced with various stimuli that mimic TCR signals. Accordingly, CHO-CD80 cells are shown to be potent costimulators of T cell activation and able to synergise with both TCR receptor agonists (anti-CD3 antibodies or superantigens) and post-receptor inducers (PMA or PMA and ionomycin (P/I)). Despite their basic purpose however these experiment revealed a number of interesting observations. Most importantly, whereas in most cases the ability of CD80 to costimulate T cell proliferation, was also accompanied by induction of at least some levels of the proliferative cytokine IL-2, the observed proliferation did not always correlate with IL-2 production. In other words, IL-2 production may only be one of the factors that regulate T cell proliferation.

The ability of T cells to proliferate better with CD80 is clearly seen by the titration experiments performed here with anti-CD3 antibodies and superantigens. In both cases the presence of CD80 allowed lower doses of these stimuli to effectively induce proliferation. These data therefore supported the concept that CD28 reduces the sensitivity of the T cells to antigenic stimulation (Viola and Lanzavecchia, 1996; Wells et al., 1997; Thompson et al., 1993). Interestingly high doses of anti-CD3 antibodies and superantigens (SEA and SEB) were able to proliferate T cells partially and independently of CD80. This agrees with other studies according to which strong antigenic stimuli do not absolutely require costimulation (Shahinian et al., 1993). However, it is also noteworthy that CD80 could act as a costimulator of these high strength TCR / CD3 agonists, but in a less potent manner. Interestingly, a similar effect was observed on T cells activated with high doses of PMA and ionomycin, whose proliferation was actually downregulated by CD80. These

observations may be explained by the fact that CD80 can also engage a second receptor on the T cells called CTLA-4 (Linsley et al., 1991b; Azuma et al., 1993a; June et al., 1994; Leach et al., 1996). This receptor is homologous to CD28 but instead of costimulating, it negatively regulates T cell activation (Walunas et al., 1996a; Krummel and Allison, 1996). Thus, as also discussed in more detail in chapter 5 of this thesis, strong TCR signals may reduce the potency of the costimulatory ability of CD80, by promoting its negative regulatory functions.

Many studies have repeatedly shown that one of the main outcomes of CD28 costimulation is the production of the proliferative cytokine IL-2 (Kuiper et al., 1994; Nunes et al., 1993; Gimmi et al., 1991). This is also suggested here, since the ability of anti-CD3 antibodies to synergise with CD80 and trigger proliferation was always accompanied by IL-2 production. Additionally proliferation by plate bound anti-CD3 antibodies was not accompanied by the production of IL-2 unless CD28 was also stimulated via CD80. Furthermore, all the inhibitors tested here (wortmannin, rapamycin and chloroquine), blocked proliferation and IL-2 production by CD3+CD80 stimulation. Clearly, these results suggested that CD28 engagement is important and possibly vital for the production of IL-2. It is less clear however, whether IL-2 production is vital for the proliferative responses that result after CD28 engagement. The fact that $IL2^{-/-}$ mice are able to respond to CD3+CD28 stimulation, suggests that other factors may substitute for IL-2 (Razi-Wolf et al., 1996; Khoruts et al., 1998) and that the costimulatory potential of CD28 is not attributed to IL-2 production alone.

In contrast to the studies obtained with anti-CD3 antibodies, the ability of CD80 to synergise with the PKC activator PMA was very potent in terms of proliferation, but not IL-2 production. Similar proliferative responses by P/I were accompanied by much higher levels of IL-2. This is a clear indication that the signals initiated by P/I or the combination of CD3+CD80 differ considerably from the ones induced by PMA+CD80. This may seem to contradict a previous report which have suggested

that CD80 can promote IL-2 production with PMA (Linsley et al., 1991a; Gimmi et al., 1991), but just like in the results presented here, whereas CD80 was able to synergise with PMA and induce high levels of proliferation, it induced relatively low levels of IL-2 when compared with other less potent or equal proliferative signals (P/I or CD3+CD80) (Linsley et al., 1991a; Gimmi et al., 1991). Thus, despite the IL-2 production, this cytokine may not be the main factor that promotes proliferation after PMA+CD80 stimulation.

The activation of human resting T cells after treatment with PMA+CD80 was also examined in terms of its sensitivity to various inhibitors. Interestingly, wortmannin which inhibits PI3K activity, could not abolish the ability of CD80 to synergise with PMA like it could prevent CD3+CD80 costimulation of proliferation and IL-2 production. Similarly CD80 also potentiates the proliferative responses of activated T cell blasts, in a largely wortmannin resistant fashion. The ability of CD80 to act alone in these cells is probably due to its synergy with ongoing signals within the activated T cells since it has been shown before to be time dependent (Edmead et al., 1996). More importantly however previous studies in our laboratory have clearly shown that CD80 stimulates proliferation but not IL-2 production in activated T cell blasts (Edmead et al., 1996), thus making it similar to the PMA+CD80 stimulation of resting T cells. Since both stimulations are also largely resistant to wortmannin, the PI3K pathway may not be involved in the proliferative effects but instead participate for the production of IL-2. The involvement of PI3K in costimulation of IL-2 production is however still controversial (Ueda et al., 1995; Collette et al., 1997; Truit et al., 1995; Crooks et al., 1995). Mutation studies on the PI3K binding site (pYMNM) of CD28 have shown that PI3K recruitment is required for IL-2 production (Kim et al., 1998a; Prasad et al., 1994; Truitt et al., 1994). In contrast however, other studies in jurkat T cells have suggested that the ability of the phorbol ester PMA to prevent PI3K recruitment by CD28 is not accompanied by a decrease in IL-2 production (Parry et al., 1996; Hutchcroft et al., 1995). Even more striking, a negative regulatory role of PI3K has been suggested in NFAT activation and IL-2

signalling in jurkat T cells (Ueda et al., 1995). Thus, the importance of PI3K in IL-2 production is far from clear. More controversy has been added by the fact that active T cells are thought to be less sensitive to wortmannin than resting T cells after CD28 signalling (data presented here and by Edmead et al., 1996). All these contradictory data suggest that the importance of PI3K in T cell activation may be dependent on the type of the T cell used, its activation state and the nature of the primary signal used to synergise with CD28. This is also supported by the results presented here, which suggested that CD3+CD80, but not PMA+CD80 signals are blocked by wortmannin. The role of PI3K in CD28 signalling and IL-2 production has therefore been under considerable debate. Additional suggested roles of PI3K downstream of CD28 are the control and regulation of cytoskeletal rearrangements (Kaga et al., 1998a), endocytosis (Cefai et al., 1998) and survival (Collette et al., 1997; Boise et al., 1995b; Noel et al., 1996; Sperling et al., 1996; Levine et al., 1997). In fact PI3K recruitment is required for receptor mediated endocytosis of CD28 although it is not yet clear if PI3K enzymatic activity is also vital for this (Cefai et al., 1998). The signals that may mediate this function are therefore still under investigation, but studies with other receptors have shown the ability of PI3K to activate Rac (Hawkins et al., 1995) which is thought to be involved in cytoskeletal rearrangements and may be vital for vesicular transportation during CD28 endocytosis (Lamarche et al., 1996; Ridley and Hall, 1992; Ridley et al., 1992). Furthermore, CD28 has been shown to regulate actin polymerisation and organisation (Kaga et al., 1998a), a vital regulatory step in intracellular trafficking. The importance of PI3K as a survival molecule is thought to be mediated by the activation of another kinase called PKB (Boise et al., 1995b; Yao and Cooper, 1995; Kauffman-Zeh et al., 1997) and the activation of anti-apoptotic proteins such as bcl-X_L (Boise et al., 1995b; Kauffman-Zeh et al., 1997). Another anti-apoptotic protein (bcl-2) is also induced by costimulatory signals but its levels are thought to be high on resting T cells as well (Noel et al., 1996) and it is therefore suggested to support the survival of resting T cells mainly (Boussiotis et al., 1997;) during cytokine / growth factor signalling (Boise et al., 1995a).

Interestingly, apart from being resistant to wortmannin proliferation of PMA+CD80 treated T cells was also found resistant to rapamycin, an inhibitor of an enzyme called target of rapamycin (TOR) that is responsible for the (direct or indirect) activation of p70S6 kinase (Price et al., 1992; Chung et al., 1992; Kunz et al., 1993). Overall these results suggest that neither PI3K nor TOR are utilised downstream of CD28 after stimulation with PMA+CD80. It must be noted however that these data do not dismiss the ability of CD28 to activate PKB independently of PI3K. In fact a recent report has suggested the ability of CD28 to act independently of PI3K and allow bcl-X_L induction (Collette et al., 1997). This survival factor may allow T cells to respond better without utilising cytokines such as IL-2.

Interestingly IL-2 is also suggested to utilise the PI3K/PKB/p70S6 kinase pathway **(diagram 3.1)** (Kirken et al., 1997; Miyazaki et al., 1995; Remillard et al., 1991). Since T cells induced by PMA+CD80 did not produce IL-2 in large amounts, it is possible that the resistance to both wortmannin and rapamycin arise partly from their independence to these signalling cascades and therefore IL-2 itself. In contrast proliferative signals induced by CD3+CD80 which produce IL-2 and are blocked by both wortmannin and rapamycin would be suggested to be more dependent on IL-2 receptor signalling. These results therefore suggested that if PI3K and TOR / p70S6 kinase are downstream of CD28 their importance in T cell activation may vary depending on the nature of the primary / synergising stimulus.

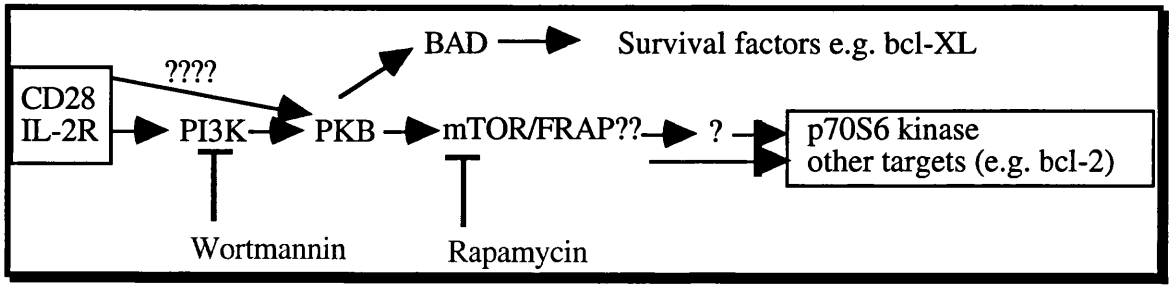

Diagram 3.1: The PI3K-PKB-p70S6kinase pathway downstream of CD28 and the IL-2R. The sites of action of wortmannin and rapamycin are also indicated.

A second lipid kinase that is thought to be induced by CD28 is aSMase (Boucher et al., 1995; Edmead et al., 1996). The studies presented here however are inconclusive about the importance of aSMase in T cell activation. Chloroquine could prevent T cell activation induced by all combinations of signals tested in both resting and activated T cell blasts. Even the costimulation independent activation via P/I was blocked by chloroquine. Although, this may suggest the importance of this enzyme in T cell signalling, chloroquine is an indirect inhibitor of aSMase and therefore not very specific. Although at the concentrations used here chloroquine is not thought to be toxic (Hedin and Thyberg, 1985) its broad action by altering the pH in the lysosomal compartments of the cell, will also affect other enzymes. Additionally, chloroquine is thought to interfere with the later stages of CD28 internalisation in late endosomes and subsequent degradation (Cefai et al., 1998). All these factors may be non specifically affecting not only CD28 signalling but also T cell activation in general.

Generally the results presented in this initial chapter show the ability of CD80 to act as a costimulator of various signals in primary T cells. Clearly however, the importance of CD80 signals is dictated by the primary stimulus and the activation state of the cell. Thus, T cells further proliferate with CD80, although this potential is decreased at high strength primary signals. Additionally, in some cases T cells proliferate with little or without the need of IL-2 production, an important proliferative cytokine. Thus, the pathways that CD28 initiates and result in proliferation may not always be linked with the production of IL-2. Concerning these pathways, PI3K and p70S6 kinase which are thought to be downstream of CD28, seem to participate in the overall costimulatory activity. Their importance however is not universal and their specific role may be determined by the nature and strength of the primary signal and the activation state of the cell. In light of the fact that *in vivo* not all antigens are the same and that each may stimulate the TCR uniquely, together with the fact that not all T cells in the periphery are in the same resting / naive state, CD80 and other CD28 ligands must play a crucial role in

determining how the cells are going to respond, proliferate and expand during an immune response.

CHAPTER 4

IL-2 INDEPENDENT T CELL ACTIVATION INDUCED BY CD28

4.1: INTRODUCTION

Data in chapter 3 suggested that the ability of CD80 to synergise with PMA in terms of proliferation is accompanied by very low levels of IL-2. In contrast, ionomycin could costimulate PMA activated T cells both in terms of proliferation and IL-2 production. This was surprising because one of the most obvious features associated with CD28 costimulation is the induction and secretion of a number of cytokines, of which IL-2 has been the best studied (Kuiper et al., 1994; Linsley et al., 1991a; Fraser et al., 1992; Nunes et al., 1993). On the other hand, T cells from IL-$2^{-/-}$ mice are still able to respond to CD3+CD28 stimulation (Razi-Wolf et al., 1996), suggesting that the action of CD28 may not be as clear cut as producing IL-2. In this respect, other studies have clearly shown that CD28 costimulation plays a role in other important functions, such as protection from cell death (Boise et al., 1995b; Noel et al., 1996; Sperling et al., 1996; Boise et al., 1995a; Daniel et al., 1997) and increasing longevity of T cell responses (Lucas et al., 1995; Levine et al., 1997) which may not be related to IL-2. Whatever the final outcome of CD28 stimulation, one widely reported feature of its signalling capability has been its relative resistance to cyclosporin A (CsA) (June et al., 1987; Osorio et al., 1998; June et al., 1989; Hess and Bright, 1991; Lu et al., 1995). CsA is a potent immunosupressant, that has been extensively used in vivo to delay, reduce or prevent immune responses (Schreiber and Crabtree, 1992; Bach, 1993; Liu, 1993). Its target is the phosphatase calcineurin (Liu, 1993; Guerini, 1997), which is activated by calcium elevation after signals from the TCR and is responsible for the translocation of the transcription factor NFAT to the nucleus (Jain et al., 1993a; McCaffrey et al., 1993b; McCaffrey et al., 1993a; Flanagan et al., 1991). However, signals downstream of CD28, after engagement with its natural ligands CD80 / CD86, do not elevate calcium levels in the cell and are therefore resistant to CsA (Linsley et al., 1991a; Gimmi et al., 1991; June et al., 1987; Lu et al., 1995).

In the studies presented in this chapter the signals initiated by PMA+CD80 were further investigated and compared with the ones induced by PMA and ionomycin (P/I). Their sensitivity to the immunosupressive drug CsA was initially investigated and the independence of IL-2 examined further by the use of IL-2 receptor blocking experiments. Additionally, in order to examine IL-2 induction in more detail, the effect of each signal on the transcription factors NF-kB, AP1 and NFAT that bind to the IL-2 gene promoter were performed. The data further support the suggestion that CD28 engagement by CD80, is capable of costimulating T cells independently of IL-2 and in a manner which is CsA resistant. Finally, several experiments are presented which attempted to determine the nature of the signal that most possibly replaces the action of IL-2 and induces proliferation after treatment with PMA+CD80.

4.2 RESULTS

4.2.1: Effect of CsA on T cell proliferation and IL-2 production.

In contrast to TCR signals (Emmel et al., 1989), CD28 signalling cascades do not involve calcium elevation and do not utilise calcineurin, making them therefore resistant to CsA (Lu et al., 1995; June et al., 1987; June et al., 1989). However, costimulation involves the synergy of signals mediated by the TCR and CD28. Thus, blocking TCR signalling via CsA also prevents the detection of the contribution from CD28. This can be clearly seen in **figure 4.1** where CD3+CD80 stimulation is very sensitive to even low concentrations of CsA. In contrast, PMA+CD80 stimulated proliferation in human resting T cells, in a fashion resistant to the immunosupressive drug CsA (**figure 4.1**). Stimulation of T cells with PMA

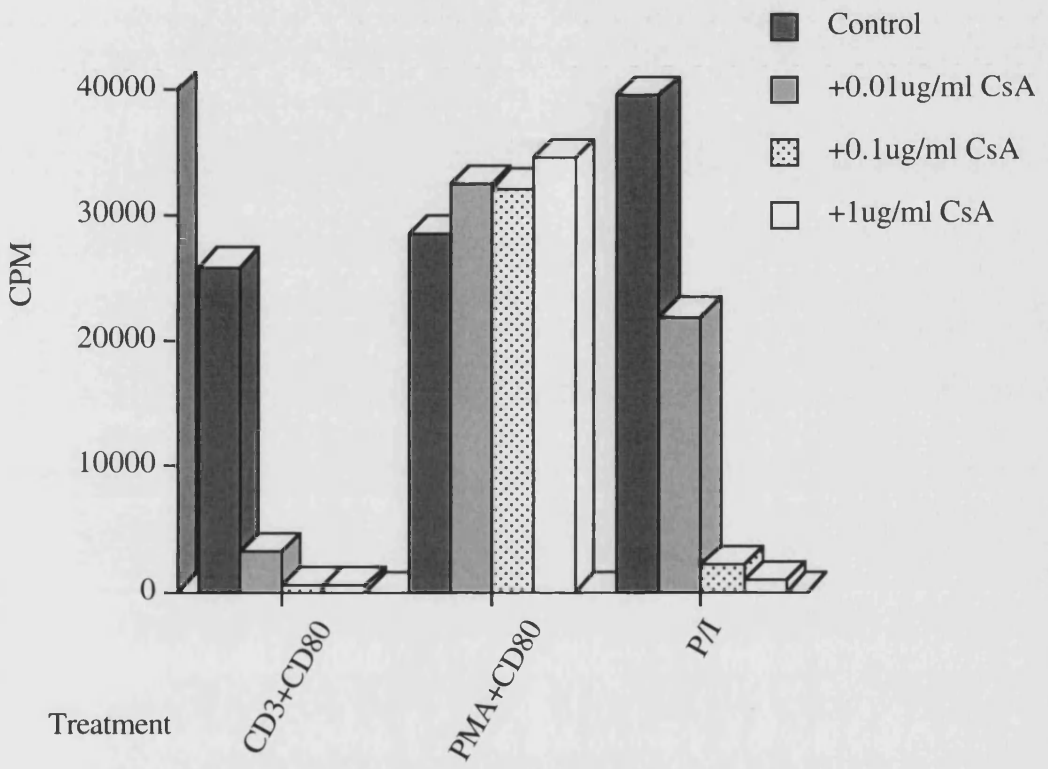

FIGURE 4.1: Variable effects of CsA on CD80 mediated T cell costimulation. Purified human resting T cells were left untreated or pre-treated with different concentrations of CsA for 30 minutes. Cells were then stimulated with soluble anti-CD3 antibody (cross-linked with anti-mouse IgG) and CHO-CD80 cells (at a ratio of 1:3 T cells) (CD3+CD80), with 5ng/ml PMA and CHO-CD80 cells (PMA+CD80) or with 5ng/ml PMA and 1μM ionomycin (P/I). Proliferation was measured at 72 hours by the incorporation of ^{3}H-thymidine during an additional 18 hour incubation.

and ionomycin (P/I) was sensitive to the drug clearly showing that the observed CsA resistance is not an effect that PMA confers alone. These results are therefore, clearly consistent with the concept that the CD28 receptor can initiate CsA resistant signals, since CD80 was essential for the CsA resistant proliferative response of PMA treated cells.

The CsA resistant action of CD28 has been previously shown by the use of antibodies for the CD28 receptor (June et al., 1987; June et al., 1989; Lu et al., 1995). In order to compare the action of ligand and anti-CD28 antibody, T cells were activated with PMA and costimulated with either ionomycin, CD80 or anti-CD28 antibodies (soluble or cross-linked with anti-mouse IgG), and the ability of each signal to induce proliferation and / or IL-2 production was measured. As shown in **figure 4.2a,** PMA was able to synergise with either CD80 or anti-CD28 antibodies and induce similar level of proliferation in a CsA resistant fashion. Interestingly anti-CD28 could costimulate T cells even when soluble antibody was used without cross-linking. In order to eliminate the possibility that anti-CD28 antibodies cross-linked during the culture period by binding to the plastic, wells were also pre-coated with FCS overnight. This did not affect the ability of anti-CD28 to induce proliferation **(figure 4.2a).** Overall it appeared that all tested CD28 agonists can synergise with PMA in a CsA resistant fashion and promote similar proliferative responses. However, when the ability of these signals to induce IL-2 was examined, substantial differences were observed **(figure 4.2b).** As also shown in chapter 3, PMA+CD80 was a weak inducer of IL-2 secretion. This low level of IL-2 production was however largely (and in some experiments completely) sensitive to CsA. Similar results were also obtained when soluble anti-CD28 antibodies were used alone to synergise with PMA. These data suggest that these signals are either able to marginally induce calcineurin via a calcium independent pathway, or elevate very low levels of calcium leading to partial activation of calcineurin. Importantly, CD80 and soluble anti-CD28 antibodies appeared to induce similar low levels of IL-2, suggesting that they stimulate CD28 similarly.

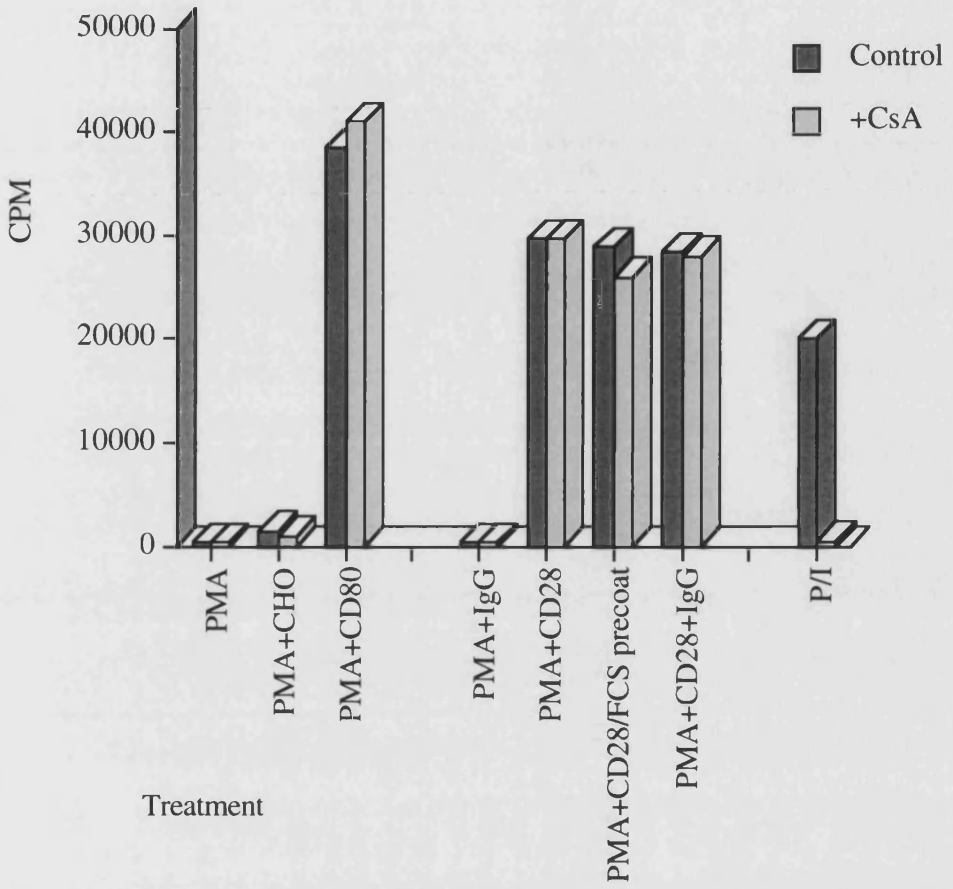

FIGURE 4.2a: Comparison of the costimulatory potential of CD80 and anti-CD28 antibodies on PMA responses of T cells and their sensitivity to CsA. Purified human resting T cells were left untreated or incubated with 1μg/ml CsA for 30 minutes. They were then stimulated with 5ng/ml PMA alone (PMA) or with CHO cells (PMA+CHO), CHO-CD80 cells (PMA+CD80), 2μg/ml soluble anti-CD28 antibodies (PMA+CD28) or 2μg/ml soluble anti-CD28 antibodies cross-linked with mouse IgG (PMA+CD28+IgG). Controls stimulations with the cross-linker alone (PMA+IgG) and soluble CD28 in wells pre-coated with FCS (PMA+CD28/FCS pre-coated) were also performed. CHO cells and CHO-CD80 cells were used at a ratio of 1:3 T cells. Proliferation was measured at 72 hours by the incorporation of ^{3}H-thymidine during an additional 18 hour incubation. The results are representative of two independent experiments.

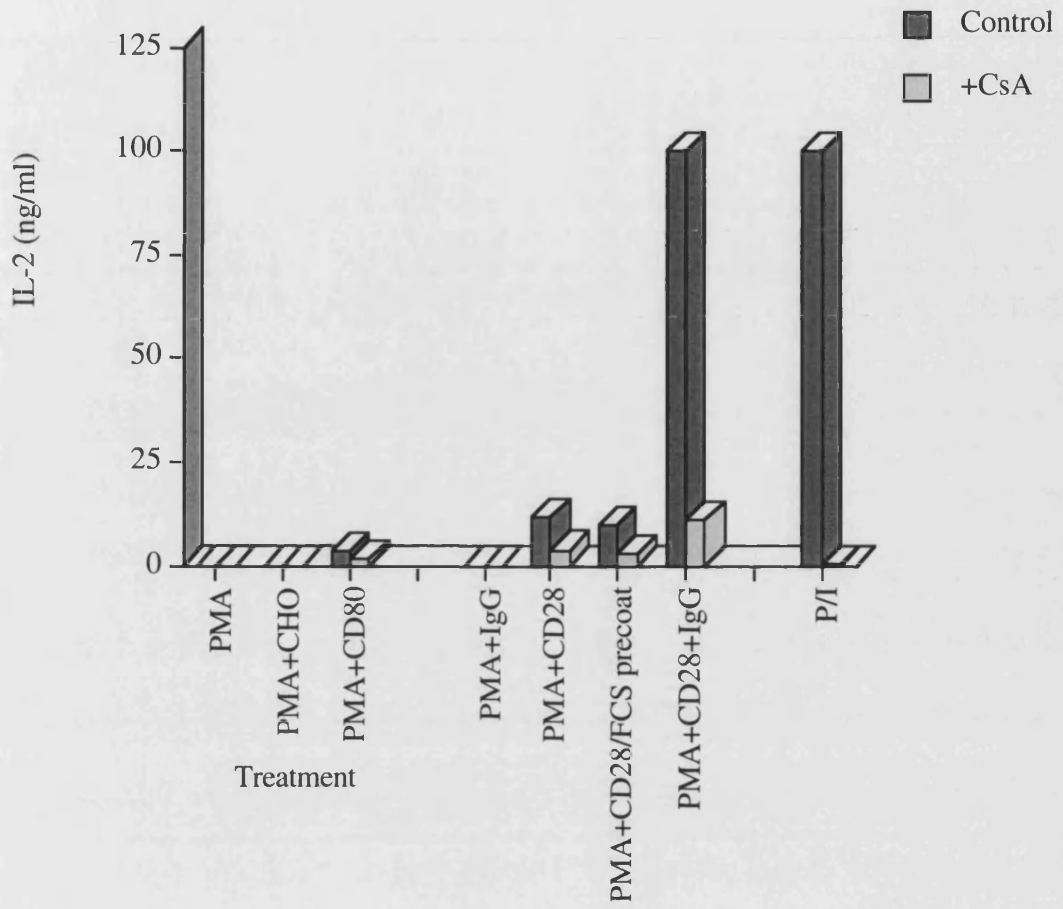

FIGURE 4.2b: Comparison of the costimulation potential of CD80 and anti-CD28 antibodies on PMA responses of T cells and their sensitivity to CsA. Purified human resting T cells were left untreated or incubated with 1μg/ml CsA for 30 minutes. They were then stimulated with 5ng/ml PMA alone (PMA) or with CHO cells (PMA+CHO), CHO-CD80 cells (PMA+CD80), 2μg/ml soluble anti-CD28 antibodies (PMA+CD28) or 2μg/ml soluble anti-CD28 antibodies cross-linked with mouse IgG (PMA+CD28+IgG). Controls stimulations with the cross-linker alone (PMA+IgG) and soluble CD28 in wells pre-coated with FCS (PMA+CD28/FCS pre-coated) were also performed. CHO cells and CHO-CD80 cells were used at a ratio of 1:3 T cells. IL-2 was quantified at 72 hours by ELISA as described in materials and methods. The results are representative of two independent experiments.

In contrast to CD80 and soluble anti-CD28 antibodies, cross-linked anti-CD28 antibodies induced high levels of IL-2, similar to the amount produced by P/I, but in both cases this was sensitive to CsA. In other words, cross-linked anti-CD28 antibodies appear to induce IL-2 via a calcineurin dependent mechanism (like ionomycin), possible due to the ability of cross-linking to elevate calcium in the cells (Ohnishi et al., 1995; Ledbetter et al., 1992). Interestingly however, whereas the induction of IL-2 by P/I was completely blocked by CsA, low levels of IL-2 remained after stimulation with PMA and cross-linked anti-CD28 antibodies in the presence of CsA **(figure 4.2b)**. It therefore seems that cross-linked anti-CD28 antibodies are also able to initiate a small signal that is calcineurin independent and can also lead to IL-2. It is not clear whether the latter is a property of cross-linked antibodies alone, or if they represent signals that are also shared after engagement with the natural ligands. However, the fact that PMA+CD80 was able in some cases to induce low levels of IL-2 even in the presence of CsA may suggest the latter. The question that now arose was whether this low level of IL-2 was significant and whether it is able to support the strong proliferative responses that are seen after these stimulations.

4.2.2: An investigation into the requirement of IL-2 in CD28 costimulation.

The ability of CD28 to mediate IL-2 expression (Linsley et al., 1991a; Fraser et al., 1992; Gimmi et al., 1991; June et al., 1987; Kuiper et al., 1994) and of IL-2 to act as a proliferative stimulus (Shibuya et al., 1992; Taniguchi and Minami, 1993; Miyazaki et al., 1995), has lead to the conclusion that the production of this cytokine is a major costimulatory effect. Despite this, the results above clearly showed that blocking the bulk of IL-2 production, via the use of CsA, does not always result in a corresponding decrease in proliferation. These data can be explained by two possibilities. The first is that, in some cases, IL-2 may be induced in excess levels, far greater than the amount required to promote T cell proliferation. Thus, even low

levels of IL-2 may be sufficient to sustain proliferation. Alternatively, the role of IL-2 may be performed by other factors, thus making the process less dependent or even completely independent of IL-2.

If the production of IL-2 is the main role of CD28, exogenous IL-2 would be predicted to largely substitute CD28 signals. To address this issue the effect of various amounts of exogenous IL-2 were examined (**Figure 4.3**). As expected, T cells alone were unable to respond to even the highest concentrations of IL-2 used. In contrast when CD3 or PMA was also given to the cells, IL-2 was able to induce a proliferative response, clearly showing the potential of this cytokine. However, the proliferative responses induced by even the highest concentrations of IL-2 together with PMA (or CD3), were not as potent as those induced by PMA (or CD3) and CD80. Significantly, the highest amount of IL-2 used in these experiments was at least 10 times higher than the levels found in the supernatants of stimulated T cells in previous experiments (**see chapter 3 at figure 3.3 and 3.5**). In this respect the inability of IL-2 to costimulate PMA (or CD3) responses was not due to the amount used. Additionally IL-2 was able to induce a proliferative response on activated T cells as later data show. Thus, although IL-2 acts in a proliferative fashion, it could not replace CD28 as a costimulator. In other words CD28 clearly performs more widespread functions than simply inducing IL-2.

The data shown in **figure 4.3** do not exclude the importance of IL-2 in costimulation since it is likely that the additional signals initiated by CD28 make IL-2 more effective, allowing low levels of IL-2 to induce proliferation. Thus, proliferation of PMA+CD80 stimulated T cells may still be dependent on IL-2, due to low amounts of IL-2 produced being subsequently consumed in an autocrine fashion. This would also render IL-2 detection more difficult. To examine this possibility, a blocking antibody to the IL-2 receptor was used. The effectiveness of this antibody was initially examined by its ability to prevent the IL-2 driven proliferative responses of SEA activated T cell blasts. Specifically, day 4 T cell blasts were treated with IL-2

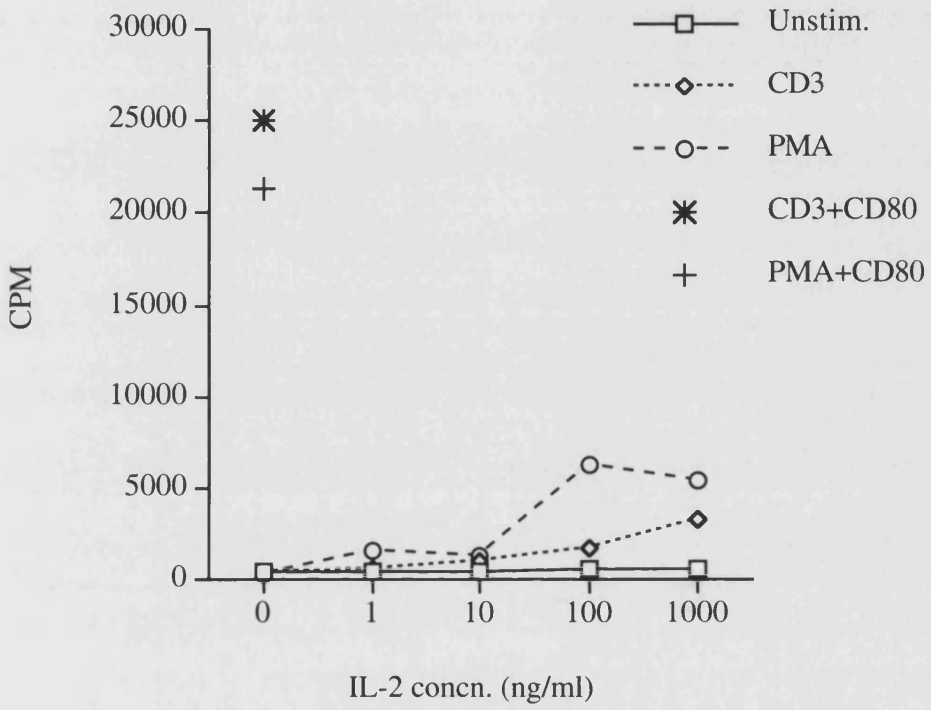

FIGURE 4.3: IL-2 as a costimulator of T cell responses. Purified human resting T cells were left unstimulated (Unstim) or incubated with 10μg/ml soluble anti-CD3 antibody (cross-linked with anti-mouse IgG) or 5ng/ml PMA. The additional effect of various concentrations of IL-2 was compared with the effect of CHO-CD80 cells (at a ratio of 1:3 T cells). Proliferation was measured at 72 hours by the incorporation of ^{3}H-thymidine during an additional 18 hour incubation. The results are representative of two independent experiments.

and left to proliferate for 24 hours, after which ^{3}H-thymidine uptake was measured for 18 hours. The effect of various concentrations of the anti-IL-2 receptor antibody is shown in **figure 4.4a.** Although complete inhibition was not seen, probably due to the large amount of IL-2 used, a clear dose dependent inhibition was observed indicating that the effect of IL-2 was being neutralised. Similar titrations of the antibody were then performed in T cells stimulated with CD3+CD80, PMA+CD80 and P/I. The results in **figure 4.4b** clearly showed that stimulation of T cells with CD3+CD80 was very sensitive to the blockade of the IL-2 receptor. In contrast stimulation of cells with PMA+CD80 was not affected by this antibody. Clearly, if any IL-2 is actually being made by the cells it does not drive their proliferation. In fact according to these results a slight increase of proliferation is observed when the IL-2 receptor is blocked, suggesting that IL-2 may actually be downregulating proliferation in this case. Finally blockade of IL-2 did not affect proliferation of T cells by P/I and again, an increase in the proliferative response is observed when the ability of IL-2 to signal via its receptor is blocked. Thus although P/I mediated signals promote IL-2 production they are not entirely dependent on it.

Collectively the results obtained here suggest that despite the proliferative potential of IL-2 on T cells, the costimulatory activity of CD80 and generally T cell activation is not always dependent on IL-2. Importantly, the results also suggest that the production of IL-2 by T cells does not always mean that the cytokine is vital for proliferation. In fact in certain cases, IL-2 may be downregulating T cell activation. This is also supported by the inflammatory phenotype of IL-2$^{-/-}$ mice (Sadlack et al., 1993) and the increased responses of NFAT1$^{-/-}$ mice against some infections (Kiani et al., 1997; Xanthoudakis et al., 1996).

4.2.3: Activation profile of cells activated with PMA+CD80.

The inability of PMA+CD80 to induce IL-2, compared to the signals induced by P/I suggested that the cells resulting after this signal may also differ in other aspects of

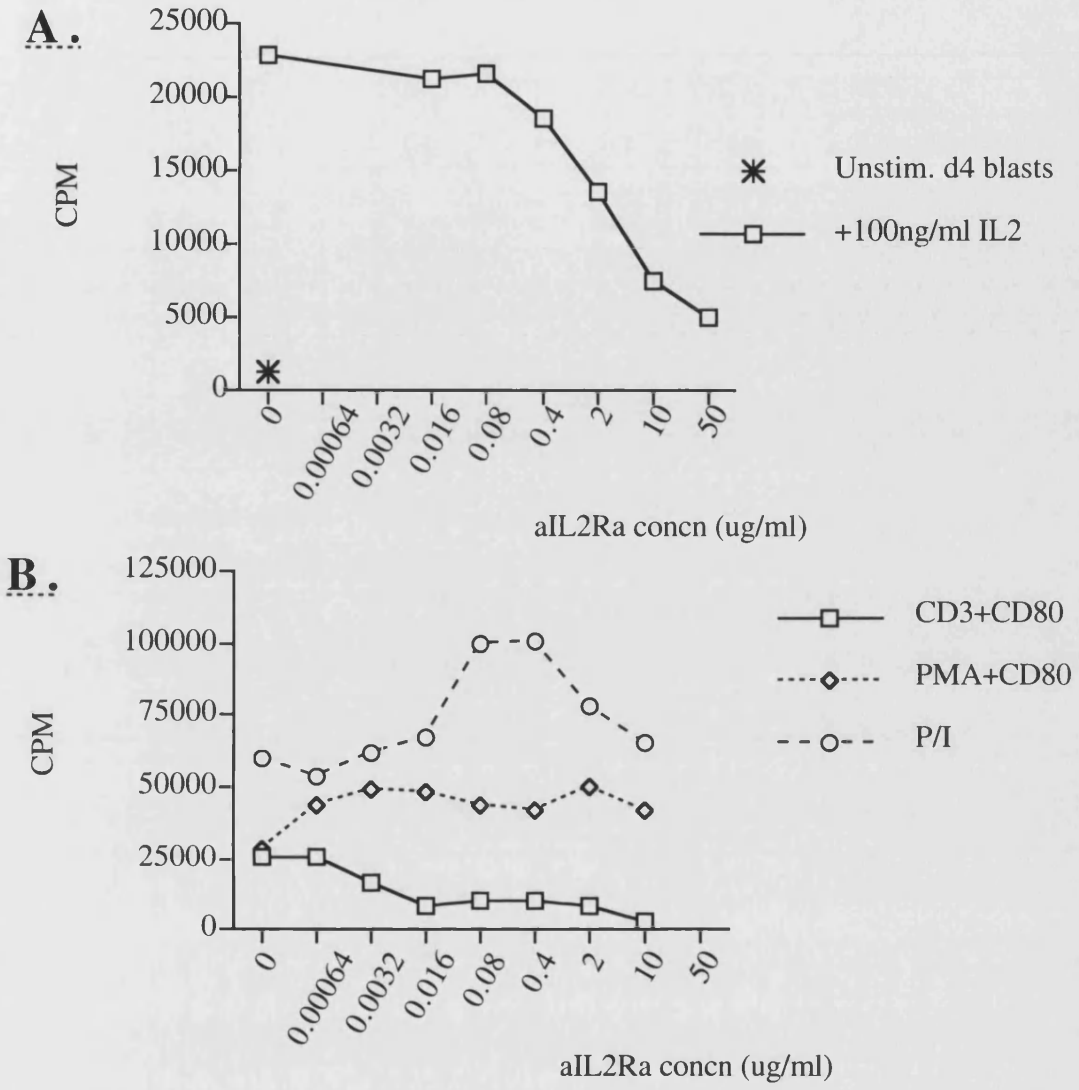

FIGURE 4.4: Effect of IL-2 receptor blockade on IL-2 dependent proliferation of activated T cells (panel A) and the costimulatory potential of CD80 (panel B). The ability of various concentrations of the anti-IL2Rα antibody to block IL-2 dependent proliferation of day 4 T cell blasts was initially examined in order to control for the effectiveness of the antibody **(panel A)**. Purified human resting T cells **(panel B)** were stimulated with soluble anti-CD3 antibodies (cross-linked with anti-mouse IgG) and CHO-CD80 cells (at a ratio of 1:3 T cells) (CD3+CD80), or with 5ng/ml PMA and CHO-CD80 cells (at a ratio of 1:3 T cells) (PMA+CD80), or with 5ng/ml PMA and 1μM ionomycin (P/I) and the effect of various concentrations of anti-IL2Rα antibody was examined on proliferation. Proliferation at **panel A** was measured at 24 hours, whereas for **panel B** at 72 hours, by the incorporation of ^{3}H-thymidine during an additional 18 hour incubation.

activation as well. It is possible for example that each type of stimulation expands a different subset of T cells. In order to address this possibility, cells stimulated with PMA, PMA+CD80 and P/I were examined for the expression of certain T cell activation markers. The results (**figure 4.5**) did not suggest any major differences amongst the cells treated with these stimulation protocols. Four days after activation cells stimulated with PMA alone do not express any CD25 although CD69 was significantly induced. The presence of CD80 or ionomycin increased the levels of CD25 dramatically in all cells. Similarly the levels of the Fas receptor four days after activation with PMA+CD80 and P/I were similar. The levels of T cell markers CD2, CD3 and CD28 were also examined on these cells. Although CD2 was present in most T cells initially, it was expressed in a higher percentage of cells after activation, possibly because of $CD2^{-ve}$ cells dying during the culture period and due to the simultaneous proliferation of $CD2^{+ve}$ cells. $CD28^{-ve}$ T cells also disappear after activation due to possibly the same reasons but also due to the ability of PMA to increase CD28 gene expression and therefore CD28 levels in the cells (Gross et al., 1992). Interestingly, the presence of CD80 in the stimulation protocol resulted in downregulation of the CD28 receptor. This is the result of the receptor mediated endocytosis of CD28 taking place after engagement with CD80 (Linsley et al., 1993; Cefai et al., 1998). The levels of CD3 which were also high in the initial population of T cells, decreased with PMA activation, as others have also suggested (Luton et al., 1997; Long et al., 1993).

Collectively, whilst only a limited survey, these data did not show any obvious differences between the two cell populations and suggested that in both cases the cells displayed the normal characteristics of activated T cells.

4.2.4: An investigation into the role of CD28 on the activation of the transcription factors that induce IL-2 gene expression.

CD28 has been suggested to induce important signalling cascades that ultimately

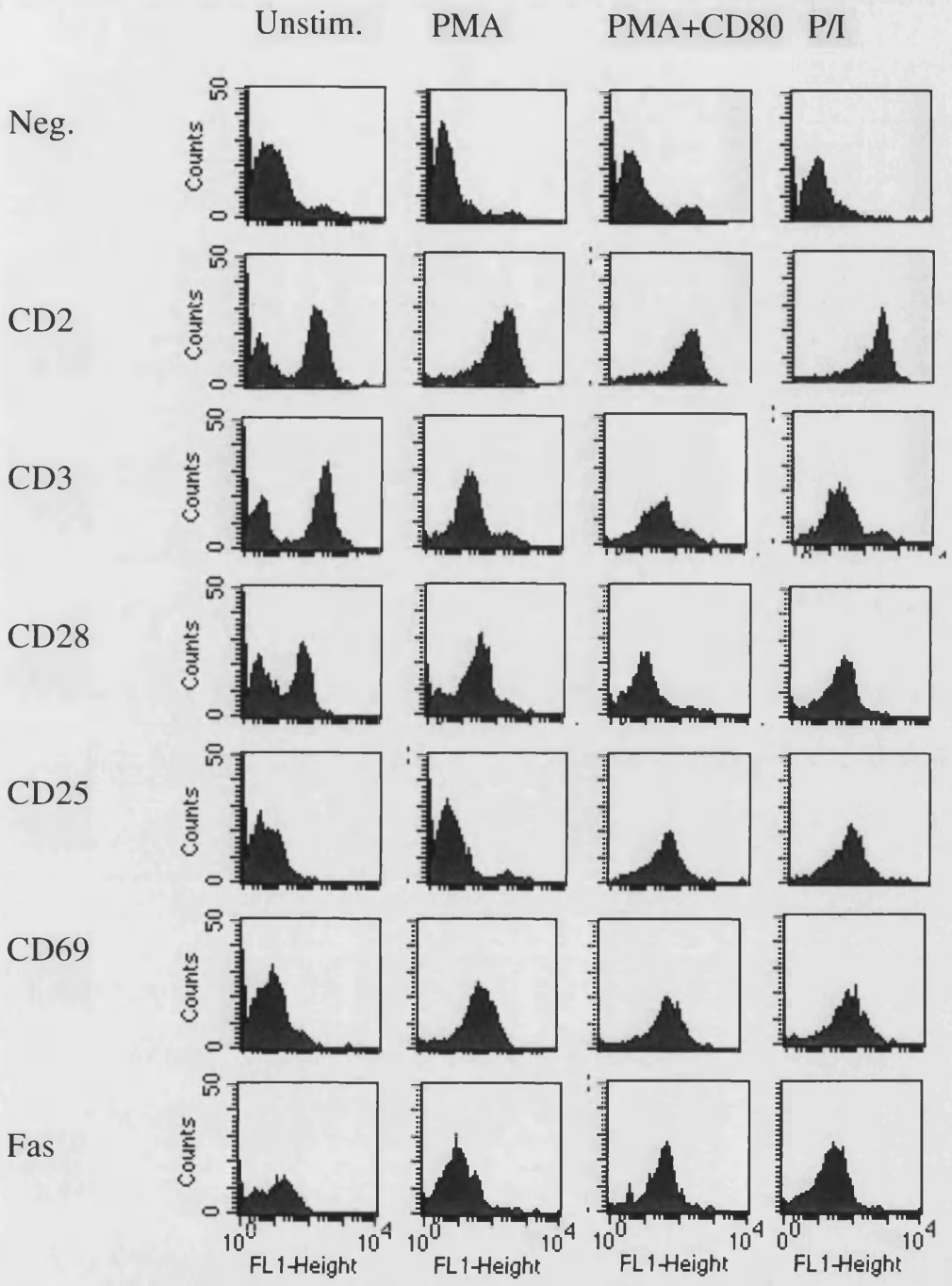

FIGURE 4.5: Surface marker levels on PMA+CD80 and P/I activated T cells. Purified human resting T cells were left unstimulated (Unstim) or were stimulated for four days with 5ng/ml PMA alone or with the additional presence of CHO-CD80 cells (at a ratio of 1:3 T cells) (PMA+CD80) or 1μM ionomycin (P/I). The cells were then stained for the surface expression of CD2, CD3, CD28, CD25 and CD69. Control staining (Neg) represents the background levels of staining induced by the secondary antibody alone.

contribute to the activation of transcription factors that participate in the expression of various genes, including IL-2 (Durand et al., 1988; Go and Miller, 1992; Fraser and Weiss, 1992; Garrity et al., 1994; Gimmi et al., 1991; Thompson et al., 1993; Freeman et al., 1993). Since PMA+CD80 was found defective in its ability to induce IL-2, it was important to understand why this happened and attempt to identify any missing signals. Examining the activation state of the transcription factors that participate in the production of the IL-2 gene, was an obvious starting point for this type of investigation, since this integrates information from a number of upstream signalling cascades that the TCR and CD28 initiate. We therefore examined the effect of PMA+CD80 on the transcription factors NF-kB, AP-1 and NFAT. At the same time, the effect induced by P/I was compared.

In order to investigate effects on the transcription factors that participate in IL-2 gene expression, electromobility gel shift assays (EMSA) were performed. This technique identifies the ability of transcription factors to enter the nucleus and bind DNA at their corresponding sites. Such studies were initially performed using jurkat T cells, which yield sufficient nuclear extracts easily, even when a small number of cells are used. In addition however, pre-activated T cell blasts and freshly purified human resting T cells were used in an attempt to examine whether these T cells behave similarly.

Despite assessing DNA binding, EMSA studies do not determine whether a transcription factor / DNA complex is actually active and able to initiate transcription. To examine this aspect of transcription luciferase reporter assays were also performed. Whereas EMSA studies can be performed in normal human T cells this is not easy for luciferase reporter assays. Transfection of the luciferase reporter constructs is difficult in T cells especially when they are in a resting state. As a result, the reporter studies shown here were performed in jurkat T cells which take up DNA with higher efficiency. The results obtained from these reporter assays for the transcription factors NF-kB, AP1 and NFAT are presented here and are

compared with the results obtained during EMSA studies on jurkat T cells. Finally experiments with human T cells are discussed and compared with results obtained from jurkat T cells.

4.2.4.1: Role of CD28 in the activation of NF-kB in jurkat T cells

NF-kB is a vital transcription factor for the expression of many genes, giving rise to proteins involved in an immune response, making it critical for the correct functioning of the immune system (Sha et al., 1995). NF-kB is a heterodimer when it binds DNA in the nucleus, consisting of various combinations of five monomers (p50, p52, p65, RelA, c-rel) (Thompson et al., 1995; Baeuerle and Henkel, 1994). Each NF-kB protein is held in the cytoplasm by the aid of ankyrin repeats whose phosphorylation and degradation unmasks nuclear localisation sequences that allow and control translocation to the nucleus (Baeuerle and Henkel, 1994; Brockman et al., 1995; Sun et al., 1996; Arrenzana-Seidedos et al., 1995).

Many signals, have been reported to induce NF-kB in the nucleus (Thompson et al., 1995). Amongst them, antibodies for the CD28 receptor have been suggested to increase and / or prolong NF-kB induction (Bryan et al., 1994; Lai and Tan, 1994; Harhaj et al., 1996). To further verify these studies the effect of CD80 examined. As **figure 4.6** shows jurkat T cells do not have any NF-kB proteins in their nucleus when they are unstimulated (lane 1) or when they are treated with CD80 alone (lane 2). Ionomycin alone was also unable to activate NF-kB DNA binding activity, but could synergise with CD80 to at least partially translocate NF-kB to the nucleus (lanes 3 and 4). In contrast to ionomycin, PMA induced NF-kB alone (lane 5) although to a lesser extent than in other experiments (see later gels). A stronger signal was however observed when PMA synergised with CD80 (lane 6). In fact the extent of NF-kB induction after these stimuli was similar to the one observed after treatment with P/I (lane 7). Interestingly, CD80 could also enhance P/I signals and increase NF-kB levels even more (lane 8). Thus, CD28 was able to maximise NF-

Lane	1	2	3	4	5	6	7	8	9	10
PMA	-	-	-	-	+	+	+	+	+	+
Iono.	-	-	+	+	-	-	+	+	+	+
CHO	+	-	+	-	+	-	+	-	+	+
CD80	-	+	-	+	-	+	-	+	-	-
xs NF-kB	-	-	-	-	-	-	-	-	+	-
xs AP1	-	-	-	-	-	-	-	-	-	+

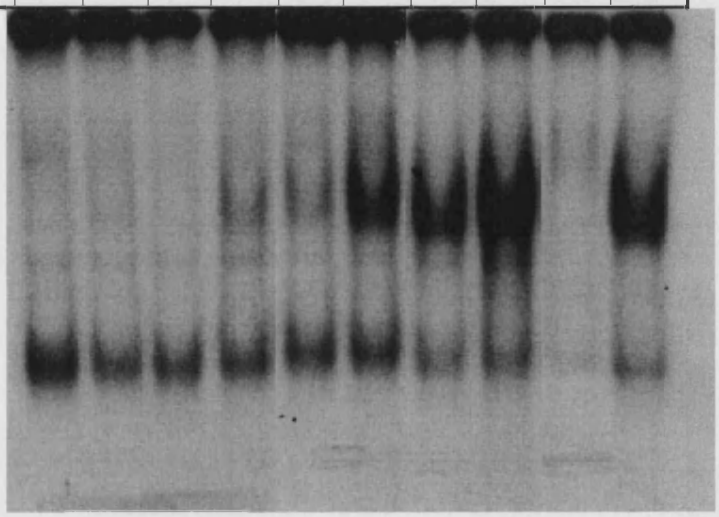

Figure 4.6: Role of CD80 on NF-kB DNA binding activity on jurkat T cells. $5x10^6$ jurkat T cells were stimulated as specified above at a concentration of 10^6 cells /ml. PMA was used at 5ng/ml, ionomycin at 1μM and CHO and CHO-CD80 cells at a ratio of 1:3 jurkat T cells. Nuclear extracts were obtained after 8 hours and were then incubated with radiolabelled NF-kB oligonucleotide and run in a gel as specified in the materials and methods. Competition assays (lanes 9 and 10) were performed with a 100 fold excess (xs) unlabelled oligonucleotide.

kB translocation to the nucleus together with signalling pathways initiated by PMA and ionomycin. Clearly, according to these results, CD28 plays an important role in the induction of NF-kB. The last two lanes on the gel in **figure 4.6** are competition assays which clearly show that excess NF-kB (lane 9), but not excess AP1 oligonucleotide (lane 10) is able to compete and prevent the detection of NF-kB / DNA complexes resulting after treatment with P/I (compare with lane 7). These assays therefore confirmed the specificity of the observed bands.

The signals that are responsible for the induction of NF-kB in T cells are under considerable debate. Several kinases and phosphatases that play crucial roles have been identified (DiDonato et al., 1997; Cao et al., 1996; Karin and Delhase, 1998; Meyer et al., 1996) and amongst them, calcineurin has been implicated (Shatrov et al., 1997; Steffan et al., 1995; Frantz et al., 1994). However, the potential of PMA+CD80 to induce NF-kB, despite their ability to act independently of calcium, does not support this. To verify the ability of CD80 to act via a different route, CsA was utilised to block the action of calcineurin. At the same time the effect of the PKC inhibitor Ro-31-8220, that is suggested to inhibit at least some of the PMA induced signals, was also investigated. As **figure 4.7** shows the ability of PMA to partially activate NF-kB DNA binding activity was further enhanced in the presence of ionomycin (lanes 1-3). This synergistic action was blocked after the addition of the PKC inhibitor Ro-31-8220 (lane 4) and the addition of CsA (lane 5). Clearly, a calcineurin dependent pathway is being utilised for the translocation of NF-kB to the nucleus after this type of stimulation. However, as also shown above, PMA could synergise with CD80 and induce NF-kB DNA binding activity and whilst the addition of Ro-31-8220 could again largely block this activation, CsA had no effect (lanes 6-8). These results further supported other reports that had implicated calcineurin as an important mediator of NF-kB activation (Shatrov et al., 1997; Steffan et al., 1995; Frantz et al., 1994) but also suggested that a second parallel and separate pathway can lead to the translocation of rel proteins in the nucleus in a manner that does not utilise calcineurin. Thus, these results verified the ability of

Lane	1	2	3	4	5	6	7	8
PMA	-	+	+	+	+	+	+	+
Iono.	-	-	+	+	+	-	-	-
CD80	-	-	-	-	-	+	+	+
Ro	-	-	-	+	-	-	+	-
CsA	-	-	-	-	+	-	-	+

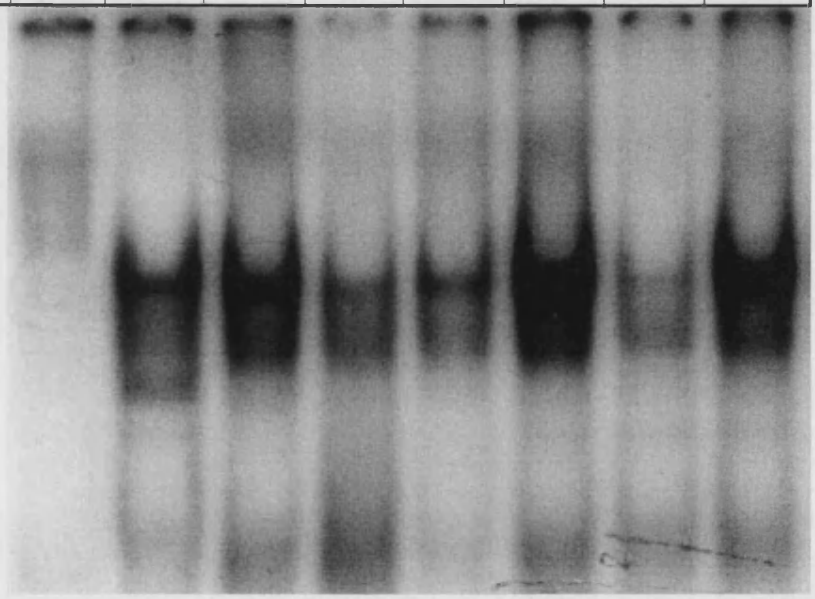

FIGURE 4.7: Effect of CsA and Ro-31-8220 (Ro) on the NF-kB DNA binding activity in jurkat T cells. $5x10^6$ jurkats T cells were left untreated or were pre-incubated with 1μg/ml CsA or 3μM Ro-31-8220 (Ro) for 30 minutes and were then stimulated as specified above at a concentration of 10^6 cells /ml. PMA was used at 5ng/ml, ionomycin at 1μM and CHO-CD80 cells at a ratio of 1:3 jurkat T cells. Nuclear extracts were obtained after 8 hours and were then incubated with radiolabelled NF-kB oligonucleotide and run in a gel as specified in the materials and methods.

CD80 to act independently of calcineurin and pinpointed NF-kB as one of its targets.

The above data suggest that PMA signals that include PKC activation, can synergise with calcineurin-dependent (initiated by the calcium elevator ionomycin), or calcineurin-independent signals (initiated by CD80) and induce NF-kB DNA binding activity. The distinctiveness of these two signals in turn, suggested that the targets of each treatment and therefore the type of NF-kB proteins that are induced may be different after each stimulus, resulting in specific NF-kB heterodimers. To examine whether the complexes resulting after each stimulation were unique, antibodies specific for certain NF-kB proteins were utilised. The addition of these antibodies to the binding reaction, prior to the addition of the radiolabelled oligonucleotide, can alter the resulting bands in the gel assays. Thus, the effect of p50, p65 and c-rel antibodies was examined on the NF-kB complexes resulting after stimulation with P/I and PMA+CD80 in jurkat T cells **(figure 4.8)**. Any low NF-kB proteins present in the nucleus of unstimulated jurkat T cells were completely supershifted by anti-p50 clearly suggesting that p50 is the main NF-kB protein found in the nucleus of unstimulated jurkat T cells. This is not surprising since p50 proteins lack transactivation domains and are therefore thought to negatively regulate gene expression (Baeuerle and Henkel, 1994). Interestingly P/I also resulted in a band that is composed of p50 since the antibody was able to supershift most of it. Noticeably however a substantial amount remained intact, suggesting that other proteins also participate. The data presented here do not indicate a role for c-rel nor p65 however. From the other NF-kB proteins RelB is not utilised in humans, thus suggesting that p52 may play a role in these complexes. Although p52 lacks transactivation domains like p50, it is able to interact with another protein called bcl-3 which is transcriptionally active (Lenardo and Siebenlist, 1994; Wulczyn et al., 1992). In fact, recent studies with p52 knockout mice have shown the importance of p52 as an NF-kB transcription factor (Caamano, at al., 1998). Although the report was not conclusive on the actual role of p52 on T cells, it is

interesting that TCR signals are suggested to activate bcl-3 (Lenardo and Siebenlist, 1994), suggesting that p52 may be utilised and activate NF-kB dependent transcription after P/I stimulation.

Contrary to ionomycin, CD80 synergised with PMA and resulted in a band that is almost completely supershifted by anti-p50 (**figure 4.8**). Although this suggested that p50 was a major constituent of these bands it does not exclude the presence of other NF-kB proteins, since as mentioned above NF-kB can be either a homodimer or a heterodimer. Even in the latter case, were only one part is made up of p50, the whole complex would be supershifted. In fact the results suggest that anti-c-rel and possibly anti-p65 could partially supershift the NF-kB complexes. This agrees with other reports that have suggested that one of the main roles of CD28 is the translocation of c-rel in the nucleus (Bryan et al., 1994; Lai and Tan, 1994; Harhaj et al., 1996). Despite this, it must be noted that the effects of these antibodies are very limited. In fact, although a similar supershift is not seen with the P/I stimulated complexes, the possibility that they result due to a non-specific interaction with the PMA+CD80 induced complex can not be ruled out. Overall however, the results suggest the presence of slightly different complexes being induced in the nucleus of jurkat T cells after each type of stimulation.

As discussed in the introduction differences in the composition of NF-kB may actually suggest differences in the transactivation potential of each complex. It was therefore important to examine the transactivation potential of the DNA / NF-kB complexes observed above. Jurkat T cells were therefore transfected with an NF-kB-luciferase (NF-kB-Luc) reporter construct. As shown in **figure 4.9** whereas untransfected cells (Neg.) did not show any luciferase levels, a basal level of NF-kB luciferase activity was seen by unstimulated cells. Interestingly, despite the ability of PMA to induce NF-kB DNA binding activity as shown by the EMSA studies above, it did not result in a similar increase of NF-kB transactivation. Ionomycin alone was also unable to enhance these basal levels but could clearly synergise with

Stimulus	Unstim.	PMA+Iono.			PMA+CD80			
rel Antibody	none	p50	none	p50	c-rel	p65	none	p50	c-rel	p65

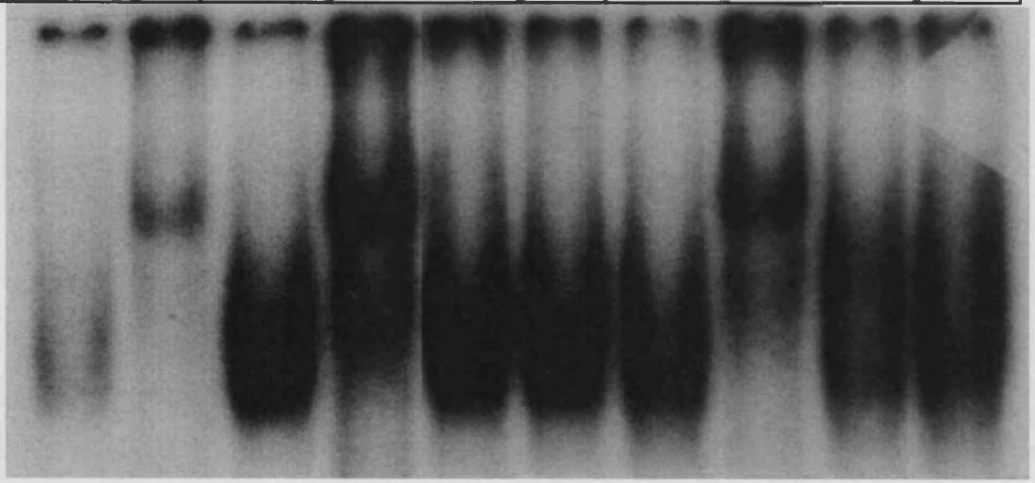

FIGURE 4.8: Role of p50, c-rel and p65 on the NF-kB DNA binding activity in jurkat T cells. $5x10^6$ jurkats T cells were stimulated as specified above at a concentration of 10^6 cells /ml. PMA was used at 5ng/ml, ionomycin at 1μM and CHO-CD80 cells at a ratio of 1:3 jurkat T cells. Nuclear extracts were obtained after 8 hours and were then incubated with 1μg/ml of the specified antibody 5 minutes prior to the addition of radiolabelled NF-kB oligonucleotide. The mixtures were run in a gel as specified in the materials and methods. The results are representative of two independent experiments.

PMA and transactivate NF-kB in a CsA sensitive fashion. Thus, as others have also suggested (Shatrov et al., 1997; Steffan et al., 1995; Frantz et al., 1994; Harhaj et al., 1996), a calcium / calcineurin dependent signal seems to play a vital role in transactivation of NF-kB. Compared to ionomycin, CD80 was able to synergise with PMA to a lesser extent, although in a clearly CsA resistant fashion. Further supporting that, although all signals together (P/I+CD80) were unable to further enhance the level of NF-kB stimulation observed by P/I, CsA was able to only decrease the transactivation level to the one resulting after activation with PMA+CD80. In other words CD80 acts with PMA to partially induce NF-kB transactivation via a mechanism that does not involve calcineurin. Clearly however, compared to its ability to translocate NF-kB to the nucleus, CD80 induces NF-kB less potently when its transcriptional activity is examined. Despite the ability of CD80 to induce DNA binding activity similar to ionomycin, it is only capable of partial transcriptional activation. This result is surprising when the supershift assays performed above are also considered, which suggested that p65 and c-rel, two of the main transcriptionally active rel proteins may partially be present on PMA+CD80, but not P/I stimulated jurkats. It is also noteworthy however that according to the supershift EMSA experiments above all complexes induced by PMA+CD80 contain p50. This suggests that a high percentage of the complex may only contain p50 homodimers which are considered transcriptionally inactive (Baeuerle and Henkel, 1994). Thus, such negatively regulatory complexes may limit the transactivation potential of NF-kB after PMA+CD80 stimulation. In contrast the lower levels of p50 after P/I stimulation may allow the remaining NF-kB proteins to initiate transcription efficiently.

4.2.4.2: Role of CD28 in the activation of AP1 in jurkat T cells

AP1 is an interesting transcription factor where CD28 is concerned because studies with CD28 antibodies have clearly suggested that it may be a major integration point between TCR / CD3 and CD28 signalling (Rincon and Flavell, 1994; Su et al., 1994; Jacinto et al., 1998; Faris et al., 1996; Woodside and McIntyre 1998).

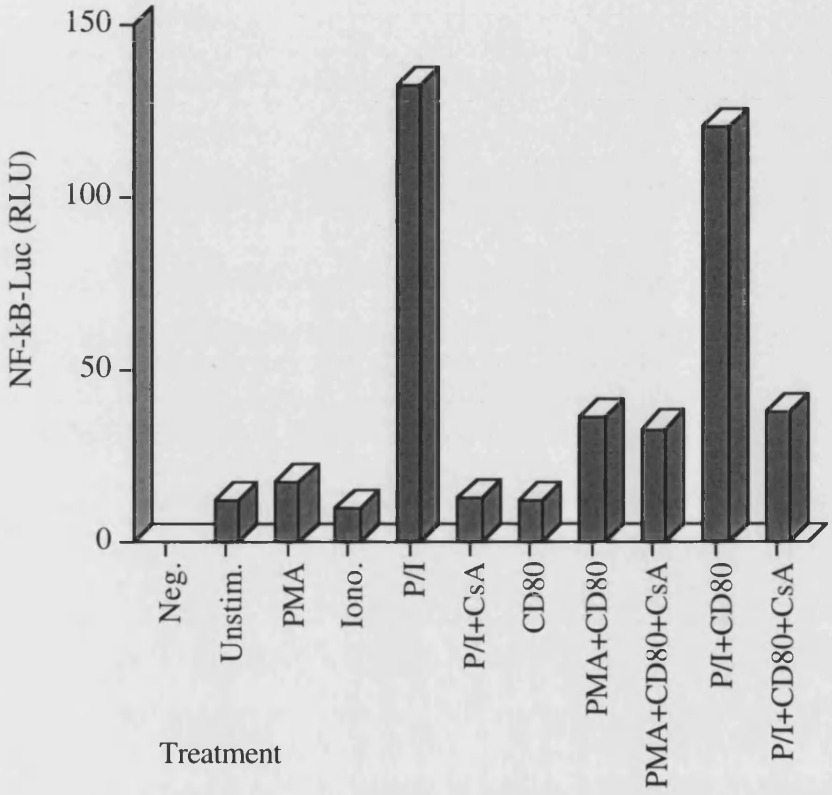

FIGURE 4.9: Transcriptional activity of NF-kB in jurkat T cells. $5x10^5$ jurkat T cells were left untreated (unstim.) or stimulated with 5ng/ml PMA (PMA), 1μM ionomycin (Iono.), 5ng/ml PMA and 1μM ionomycin (P/I), CHO-CD80 cells (CD80), 5ng/ml PMA and CHO-CD80 cells (PMA+CD80) or 5ng/ml PMA with 1μM ionomycin and CHO-CD80 cells (P/I+CD80). CHO-CD80 cells were used at a ratio of 1:3 jurkat T cells. The effect of CsA was determined by pretreating the cells for 30 minutes with 1μg/ml CsA. Cytoplasmic extracts were prepared after 12 hours and luciferase activity was determined as specified in materials and methods.

Additionally, prevention of anergy, an effect clearly mediated by CD28 (Harding et al., 1992; Gimmi et al., 1993; Mueller et al., 1989; Tan et al., 1993; Galvin et al., 1992) is highly dependent on AP1 activity (Fields et al., 1996; Carmella et al., 1996; Woodside and McIntyre 1998). Studies were therefore performed to verify the role of CD28 on AP1 after engagement with its natural ligand CD80. The ability of CD80 to induce AP1 DNA binding activity was initially examined via EMSA studies. The results in **figure 4.10** demonstrated that CD80 was unable to act alone and increase AP1 DNA binding activity (lane 2). Ionomycin was equally unable to induce AP1 at high levels and only when it synergised with CD80 low levels of AP1 were detected (lanes 3 and 4). In contrast, PMA alone was able to partially induce AP1 DNA binding activity (lane 5) but interestingly no additional effect of CD80 was observed (lanes 6). An even stronger AP1 complex was seen when PMA synergised with ionomycin (lane 7), an effect that was also not increased by CD80 (lane 8). Overall, these results surprisingly suggested that CD28 does not influence the DNA binding ability of AP1.

In examining the transactivation potential of AP1 **(figure 4.11)**, PMA was found able to induce high AP1 luciferase activity. Ionomycin on the other hand was unable to induce AP1, but could further potentiate PMA stimulation. Interestingly, despite the limited ability of CD80 to synergise with PMA and induce AP1 binding activity, it could substitute for ionomycin and result in an equal enhancement of AP1 transcriptional activity. Additionally this synergy was resistant to CsA. Clearly, CD80 seems to play an important role in the regulation of AP1, by increasing its transactivation potential. As others have suggested (Hibi et al., 1993; Su et al., 1994) this action of CD28 is most possibly mediated by the activation of JNK, the kinase responsible for c-jun transactivation. However, the results also suggest that the signals that PMA initiates and possibly represent part of the TCR pathways are able to induce AP1 in high levels without the need of costimulation. To further examine this possibility jurkat T cells were stimulated with anti-CD3 antibodies instead of PMA or P/I. The results in **figure 4.12** suggested that compared to PMA, CD3 was

Lane	1	2	3	4	5	6	7	8
PMA	-	-	-	-	+	+	+	+
Iono.	-	-	+	+	-	-	+	+
CHO	+	-	+	-	+	-	+	-
CD80	-	+	-	+	-	+	-	+

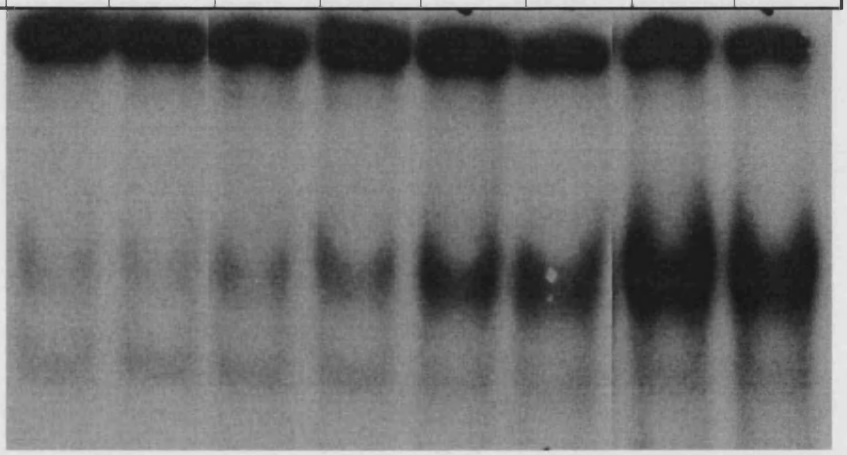

FIGURE 4.10: Effect of CD80 on AP1 DNA binding activity on jurkat T cells. $5x10^6$ jurkats T cells were stimulated as specified above at a concentration of 10^6 cells /ml. PMA was used at 5ng/ml, ionomycin at 1μM and CHO-CD80 cells at a ratio of 1:3 jurkat T cells. CHO-CD80 cells were used at 1:3 ratio to jurkat T cells, PMA was used at 5ng/ml, ionomycin at 1μM. Nuclear extracts were obtained after 8 hours and were then incubated with radiolabelled AP1 oligonucleotide and run in a gel as specified in the materials and methods.

less efficient in transactivating AP1. The additional presence of CD80 however was able to synergise very effectively and fully activate AP1. Clearly, CD3 signal can induce AP1, but the effect of PMA seems to be over exaggerated.

4.2.4.3: Role of CD28 in the activation of NFAT and the whole IL-2 promoter in jurkat T cells

A third transcription factor that is vital for the expression of not only IL-2, but of other cytokine genes as well, is a composite transcription factor that contains AP1 proteins together with a protein called NFAT1 or NFATp (Jain et al., 1993a; Rao et al., 1997; Northrop et al., 1993; Boise et al., 1993a). Both are required for a fully active NFAT complex to be formed (Rao, 1997; Jain et al., 1993a). Contrary to AP1 proteins that reside in the nucleus, NFAT1 is present in the cytoplasm and is translocated to the nucleus after activation in a calcium dependent manner (McCaffrey et al., 1993b; Jain et al., 1993a; Flanagan et al., 1991). Luciferase assays were performed in order to examine the activation of this transcription factor in jurkat T cells. As shown in **figure 4.13** a strong NFAT response followed stimulation by PMA and ionomycin but not by either alone. This effect was completely blocked by CsA, clearly supporting the importance of calcineurin. However, in contrast to the results for NF-kB and AP-1, PMA+CD80 was unable to activate the NFAT reporter construct. Even when P/I were used together, CD80 was unable to further enhance the transcriptional activity of NFAT. Thus, as clearly established by others (McCaffrey et al., 1993b; Jain et al., 1993a; McCaffrey et al., 1993a; Flanagan et al., 1991), a calcium / calcineurin pathway is vital for the transactivation of NFAT presumably to translocate NFAT1 from the cytoplasm to the nucleus. Additionally, these results suggest that the partial ability of anti-CD28 antibodies to activate NFAT in a CsA resistant fashion (Ghosh et al., 1996; Lyakh et al., 1997; Nebl et al., 1998), may not be shared by the natural ligand CD80.

The results above suggest that the inability of PMA+CD80 to stimulate NFAT may well be the reason for its inability to induce IL-2. To further verify this, luciferase

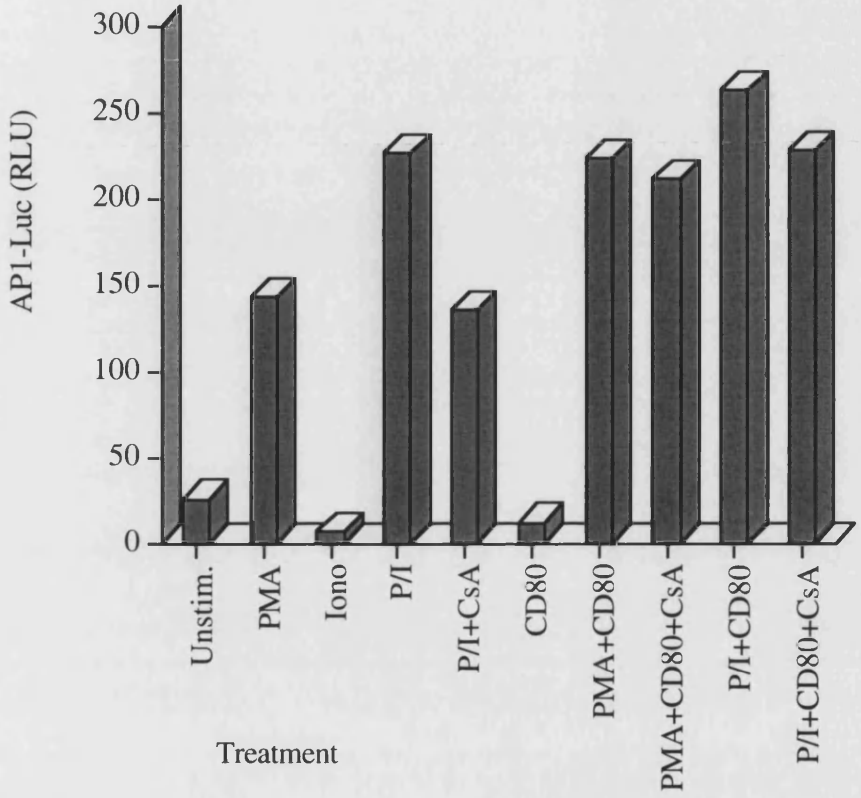

FIGURE 4.11: Transcriptional activity of AP1 in jurkat T cells. $5x10^5$ jurkat T cells were left untreated (unstim.) or stimulated with 5ng/ml PMA (PMA), 1μM ionomycin (Iono.), 5ng/ml PMA and 1μM ionomycin (P/I), CHO-CD80 cells (CD80), 5ng/ml PMA and CHO-CD80 cells (PMA+CD80) or 5ng/ml PMA with 1μM ionomycin and CHO-CD80 cells (P/I+CD80). CHO-CD80 cells were used at a ratio of 1:3 jurkat T cells. The effect of CsA was determined by pretreating the cells for 30 minutes with 1μg/ml CsA. Cytoplasmic extracts were prepared after 12 hours and luciferase activity was determined as specified in materials and methods.

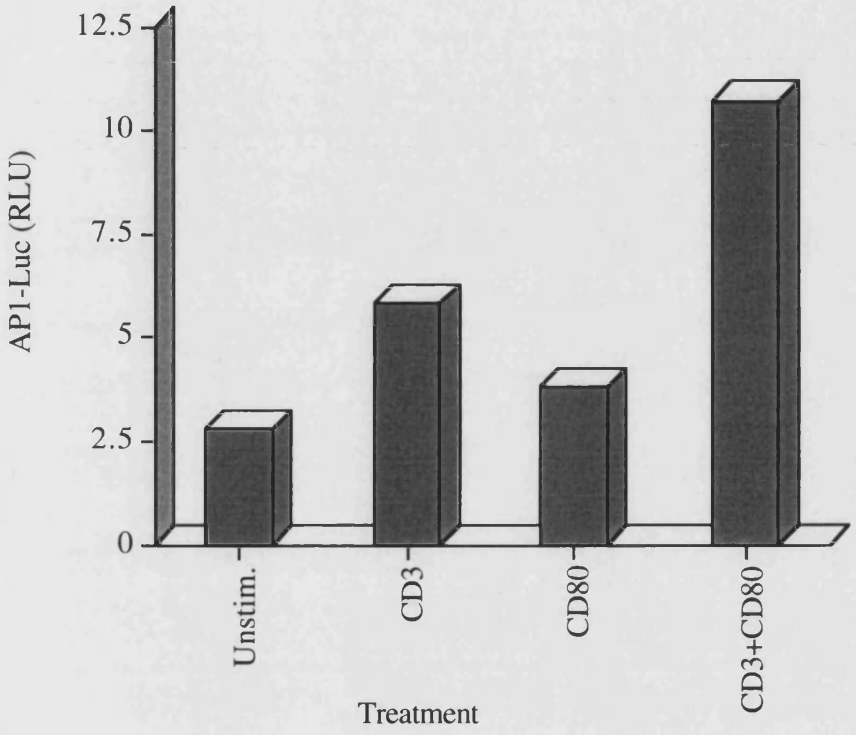

FIGURE 4.12: Transcriptional activity of AP1 in jurkat T cells. $5x10^5$ jurkat T cells were left untreated (Unstim.) or stimulated with 10μg/ml soluble anti-CD3 antibody (cross-linked with mouse IgG) (CD3), CHO-CD80 cells at a ratio of 1:3 jurkat T cells (CD80) or both together (CD3+CD80). Cytoplasmic extracts were prepared after 12 hours and luciferase activity was determined as specified in materials and methods. The results are representative of two independent experiments.

assays were also performed with reporter constructs regulated by the whole IL-2 promoter. Similar results were obtained to those with NFAT (**figure 4.14**). Some low levels of IL-2 activation induced by PMA+CD80 were blocked by CsA, suggesting that this is due to calcineurin involvement. Importantly however, CD80 was in this case able to further enhance the level of IL-2 promoter transcriptional activity mediated by P/I. This effect is probably due to the ability of CD80 to participate in the induction of NF-kB and AP1 as shown above. Interestingly, NF-kB and AP1 have recently been characterised as the main factors that bind the CD28RE, an element in the IL-2 promoter that depends on CD28 stimulation (McGuire and Iacobelli, 1997). However the effect of CD28 on these transcription factors is only visible in the final outcome (i.e. IL-2 induction), when the third transcription factor (i.e. NFAT) is also induced. CsA was able to completely inhibit IL-2 activation by presumably abolishing the induction of NFAT. The absence of the latter would render the whole IL-2 promoter highly inefficient.

The levels of NFAT and IL-2 activation were also examined after stimulation with CD3+CD80 (**figure 4.15**). Since this type of stimulus produces IL-2 in T cells, it was also expected to induce both NFAT and the whole IL-2 promoter in jurkat T cells. The results in **figure 4.15a** show that NFAT activation was induced by anti-CD3 antibodies alone, probably due to the ability of TCR signals to translocate NFAT via its calcium / calcineurin pathway and induce AP1 via its calcium independent signals (as suggested by the effect of PMA and CD3 above). CD80 was found able to further increase NFAT activity possibly due to its ability to potentiate the induction of AP1. Finally, when the whole IL-2 promoter was examined, activity was highly dependent on CD28 since only when CD80 was utilised did CD3 induce the IL-2 luciferase reporter (**figure 4.15b**). Thus, the presence of the calcium signals induced by the anti-CD3 antibodies allows CD80 to costimulate IL-2 gene expression.

Overall the data obtained here further supported the results obtained with "normal"

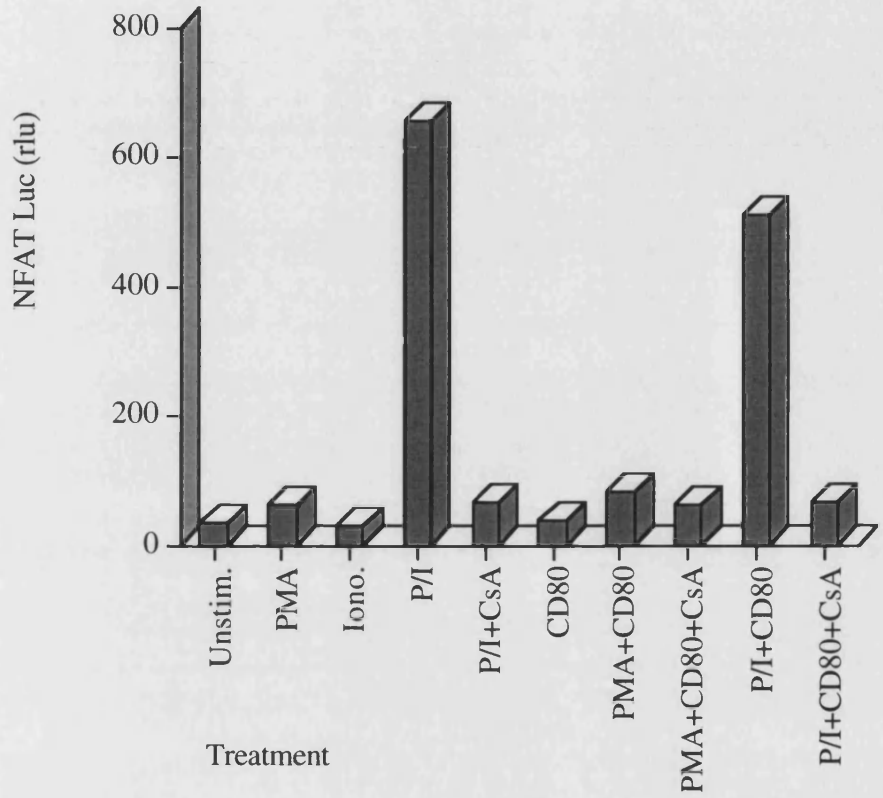

FIGURE 4.13: Transcriptional activity of NFAT in jurkat T cells. $5x10^5$ jurkat T cells were left untreated (Unstim.) or stimulated with 5ng/ml PMA (PMA), 1μM ionomycin (Iono.), 5ng/ml PMA and 1μM ionomycin (P/I), CHO-CD80 cells (CD80), 5ng/ml PMA and CHO-CD80 cells (PMA+CD80) or 5ng/ml PMA with 1μM ionomycin and CHO-CD80 cells (P/I+CD80). CHO-CD80 cells were used at a ratio of 1:3 jurkat T cells. The effect of CsA was determined by pretreating the cells for 30 minutes with 1μg/ml CsA. Cytoplasmic extracts were prepared after 12 hours and luciferase activity was determined as specified in materials and methods.

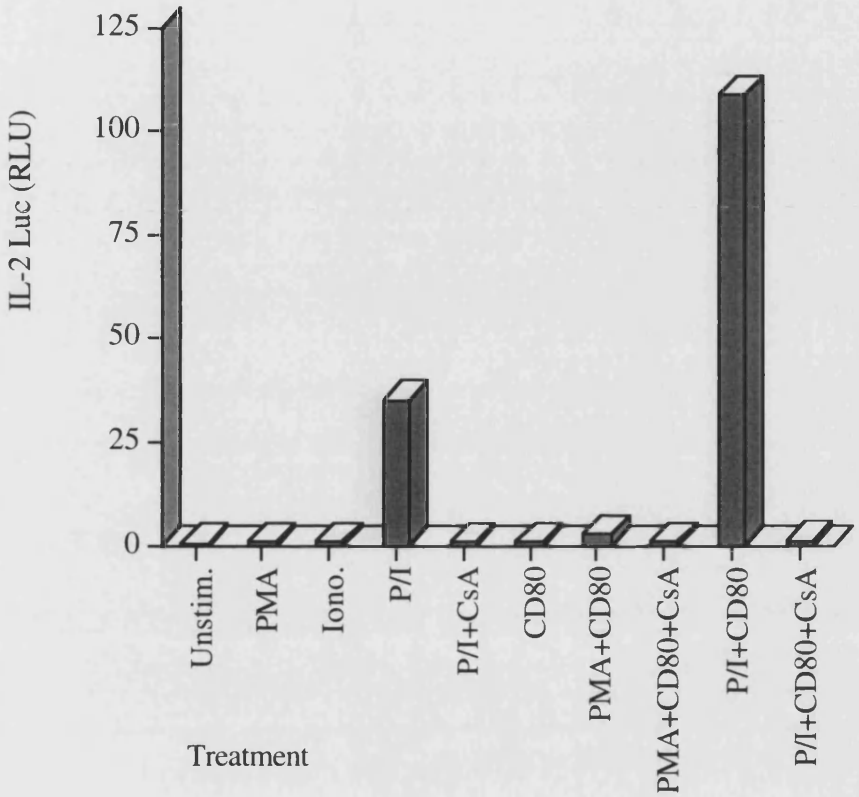

FIGURE 4.14: Transcriptional activity of the whole IL-2 promoter in jurkat T cells. $5x10^5$ jurkat T cells were left untreated (unstim.) or stimulated with 5ng/ml PMA (PMA), 1μM ionomycin (Iono.), 5ng/ml PMA and 1μM ionomycin (P/I), CHO-CD80 cells (CD80), 5ng/ml PMA and CHO-CD80 cells (PMA+CD80) or 5ng/ml PMA with 1μM ionomycin and CHO-CD80 cells (P/I+CD80). CHO-CD80 cells were used at a ratio of 1:3 jurkat T cells. The effect of CsA was determined by pretreating the cells for 30 minutes with 1μg/ml CsA. Cytoplasmic extracts were prepared after 12 hours and luciferase activity was determined as specified in materials and methods.

T cells which suggested that PMA+CD80 signals do not produce significant amounts of IL-2 and induce proliferation independently of IL-2. Furthermore, the results suggested that CD28 participates in IL-2 transcription only when TCR signals are fully induced (e.g. via P/I or anti-CD3 antibodies). In contrast, in the absence of calcium and specifically calcineurin activation (e.g. PMA stimulation) the effects of CD28 on NF-kB and AP1 are insufficient to promote IL-2 production.

4.2.4.4: Transcription factors activated by CD28 in normal human T cells.

The results obtained with the jurkat T cells clearly supported the concept that PMA+CD80 was able to induce only limited levels of IL-2 since the signals initiated by these two stimuli were able to synergise and activate NF-kB and AP-1 but not NFAT and failed to activate the whole IL-2 promoter. Despite this, jurkat T cells are not physiological in many respects, including their activation and metabolic state, especially when compared with resting T cells. The roles of the above transcription factors in normal human T cells were therefore examined and EMSAs were performed with purified human T cells to determine any differences in the activation of transcription factors in jurkat and normal T cells.

4.2.4.4a: NF-kB DNA binding activity in human T cells.

Studies for the NF-kB transcription factor with previously activated T cells are represented in **figure 4.16.** A basal level of NF-kB binding activity is observed in unstimulated T cells (lane 1-band B) which is increased by treatment with PMA alone (lane 2). Interestingly, the additional presence of ionomycin results in a more intense NF-kB / DNA complex that is also characterised by its slower migration in the gel (band A) (compare lanes 2 and 3). This suggests that new NF-kB dimers of higher molecular weight result with P/I or that the same dimers may be modified after the additional presence of ionomycin. Whereas the activation of NF-kB by P/I was sensitive to CsA (lane 4), when CD80 was used in synergy with PMA, band A also appeared, but this time in a CsA resistant fashion (lanes 5 and 6). The induction of this band was however only partial compared to the one resulting after P/I and

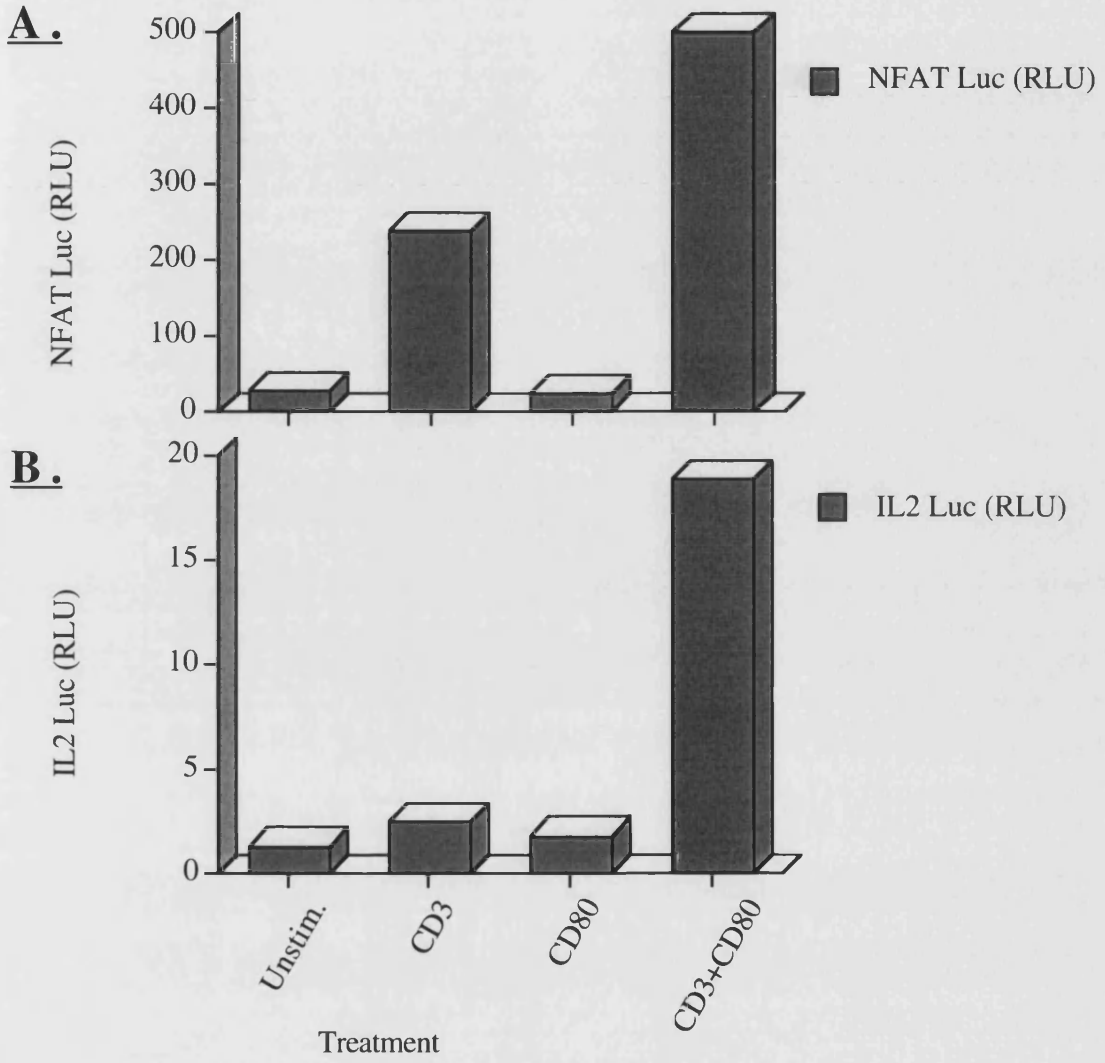

FIGURE 4.15: Transcriptional activity of NFAT and the whole IL-2 promoter in jurkat T cells stimulated with CD3 and CD80. $5x10^5$ jurkat T cells were left untreated (Unstim.) or stimulated with 10μg/ml soluble anti-CD3 antibody (cross-linked with mouse IgG) (CD3), CHO-CD80 cells at a ratio of 1:3 jurkat T cells (CD80) or both together (CD3+CD80). Cytoplasmic extracts were prepared after 12 hours and luciferase activity was determined as specified in materials and methods. The results are representative of two independent experiments.

some levels of the lower band (band B), remained intact. Finally the combination of all signals together (P/I+CD80) resulted in a band, very similar to P/I, although of less intensity (lane 7-compare with 3). Not surprisingly CsA blocked the action of ionomycin and returned the levels of NF-kB to the ones observed by PMA+CD80, with intermediate levels of both bands A and B (lane 8).

The presence of various bands after different treatments suggested the presence of differences in the NF-kB complexes after each stimulation. There are various possibilities that might explain the presence of bands with variable molecular weights. Firstly, rel proteins that bind DNA after stimulation with P/I may be modified (e.g. by phosphorylation or ubiquitination). Alternatively, specific proteins that also regulate transcription may interact with P/I induced NF-kB complexes, just like the co-activators that are suggested to regulate AP1 transcriptional activity (Claret et al., 1996). Most possibly however, the different bands observed in the gels are simply the result of variable composition of rel proteins. To examine the specificity of these bands as well as the possibility that they are the result of different NF-kB complexes, competition and supershift assays were performed as before (**Figure 4.17**). The complex resulting after treatment with P/I+CD80+CsA was used as a control, since it resulted in the presence of both bands A and B. The specificity of each band was initially examined via the aid of competition assays. As expected excess AP1 oligonucleotide had no effect (lane 3) but excess unlabelled NF-kB was able to prevent the appearance of both bands (lane 2). However, some levels of band B remained untouched. This may have resulted because the competitor was not given enough time to act, since it was added to the binding mixture only a few seconds before the labelled oligonucleotide in this particular case. Alternatively this may simply mean that part of band B is not specific for NF-kB. However, further specificity studies with the antibodies for NF-kB proteins suggest that the former is more possible. The anti-p50 antibody was very potent in its ability to detect NF-kB complexes and was able to completely supershift band B (lane 4). In fact, if anything, it left some low levels of the band A untouched. In

contrast, the anti-p65 antibody had no effect on the DNA binding activity of NF-kB (lane 5). The final NF-kB antibody for c-rel, was able to partially interfere with the binding ability of both bands in the gel (lane 6), suggesting that it may be participating in both complexes.

As with jurkat T cells, the supershift assays clearly showed the specificity of the complexes. A third band (band C) is suggested to be non specific from the fact that both excess NF-kB and excess AP1 can compete it. A fourth band (band D-seen better in **figure 4.16**), is however an additional possible NF-kB protein. Although specificity controls were not performed on this band, its complete disappearance coincides with the induction of band A (compare unstimulated-lane 1 with P/I-lane 3 on **figure 4.16**). Interestingly the stimulation that mainly results in band A (i.e. P/I), was also the one that according to the luciferase assays performed in jurkat T cells was more able to transactivate NF-kB (**see figure 4.9**). If this is also true in T cells, the complex in band A should contain a higher ratio of active (i.e. c-rel, RelA, RelB) versus inactive (i.e. p50 and p52) rel proteins, thus making it more transcriptionally active. The ability of p50 to completely supershift the band B, but leave some low levels of the band A intact **(see figure 4.17)** may support this, since it suggests that other rel proteins are more abundant in band A (the P/I induced complex). Interestingly, the results obtained from jurkat T cells above (see figure 4.8) were not very different since, although only one band was observed after P/I stimulation, it was supershifted less with the p50 antibody compare to the one induced by PMA+CD80.

The levels of NF-kB DNA binding activity were also examined on freshly purified human resting T cells. Interestingly, a basal level of NF-kB activity was detected in unstimulated cells **(Figure 4.18).** The addition of PMA was able to enhance these levels alone, but the presence of ionomycin further potentiated this effect (lanes 1-3). Again, this was accompanied by a shift of the band further up in the gel. CsA was able to prevent the shift and also decrease the overall level of NF-kB (lanes 3

Lane	1	2	3	4	5	6	7	8
Stimulus	None	PMA	P/I		PMA+ CD80		P/I+ CD80	
CsA	-	-	-	+	-	+	-	+

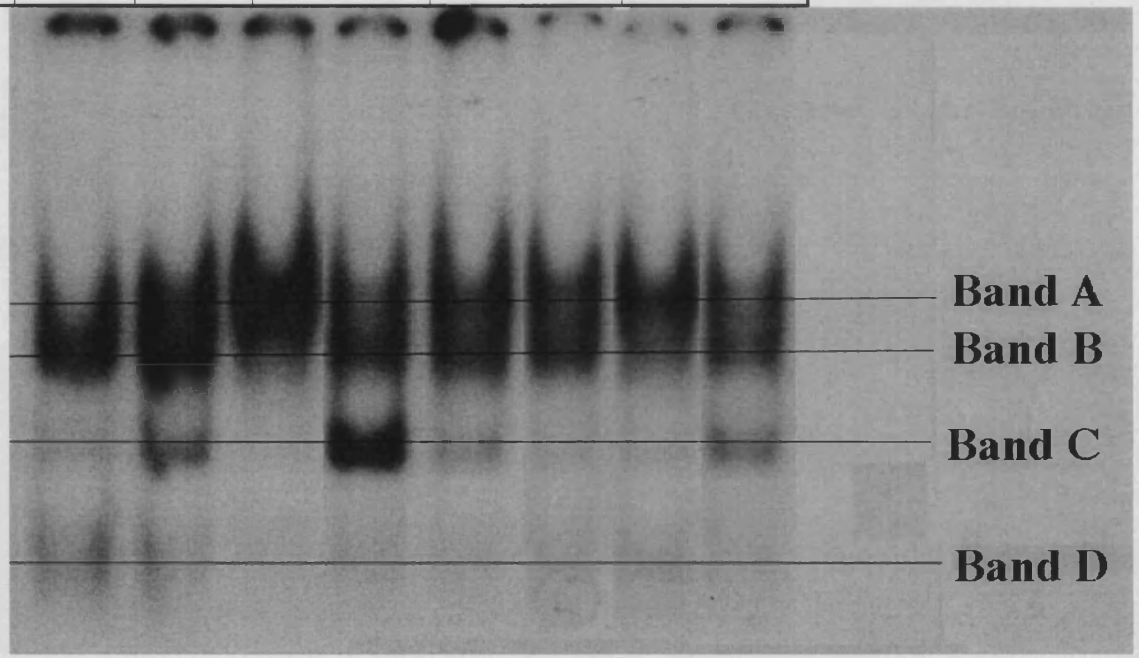

FIGURE 4.16: NF-kB DNA binding activity on pre-activated (resting) T cell blasts. 10^7 T cells blasts were stimulated as specified above at a concentration of $2x10^6$ cells /ml. PMA was used at 5ng/ml, ionomycin at 1μM and CHO-CD80 cells at a ratio of 1:3 jurkat T cells. CsA treated cells were pre-incubated for 30 minutes with 1μg/ml of the inhibitor. Nuclear extracts were obtained after 8 hours and were then incubated with radiolabelled NF-kB oligonucleotide and run in a gel as specified in the materials and methods.

Lane 1: Control (P/I+CD80+CsA stimulated T cell blasts)

Lane 2: Plus excess NF-kB

Lane 3: Plus excess AP1

Lane 4: Plus anti-p50 antibody

Lane 5: Plus anti-p65 antibody

Lane 6: Plus anti-c-rel antibody

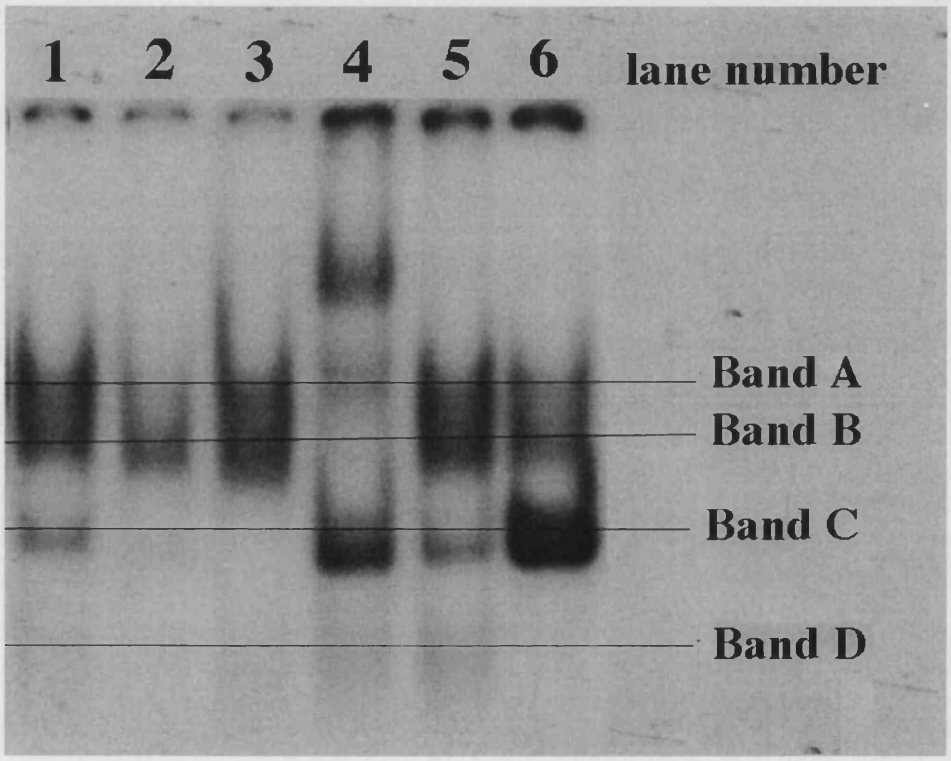

FIGURE 4.17: Role of p50, p65 and c-rel on NF-kB DNA binding activity of pre-activated (resting) T cell blasts. 10^7 T cells blasts were stimulated with 5ng/ml PMA, 1μM ionomycin, CHO-CD80 cells (at a ratio of 1:3 jurkat T cells) and 1μg/ml CsA (lane 8 of figure 4.15). Nuclear extracts were obtained after 8 hours and were then incubated with 1μg/ml of the above antibodies prior to the addition of the radiolabelled NF-kB oligonucleotide and run in a gel as specified in the materials and methods. Competition assays (lanes 2 and 3) were performed with a 100 fold excess unlabelled oligonucleotide.

and 4). CD80 was also able to enhance the ability of PMA to induce NF-kB DNA binding activity (in a CsA resistant fashion) but could not result in a similar shift (lanes 5 and 6). The specificity of the obtained complexes is clearly seen by the competition assays since the complex resulting after activation of human resting T cells with PMA+CD80 is competed by excess unlabelled NF-kB (lane 7) but not AP1 (lane 8) oligonucleotide.

Overall, as other reports have also suggested (Bryan et al., 1994; Harhaj et al., 1996; Lai and Tan, 1994) the data obtained from human T cells further supported the idea that PMA+CD80 is a potent activator of NF-kB, but that the resulting complexes differ from the ones initiated by P/I. Collectively, it is suggested that a signal initiated by ionomycin must be important for the transactivation of NF-kB. In this respect, calcineurin has been suggested to participate in the phosphorylation state of IkBα (Frantz et al., 1994; Shatrov et al., 1997; Steffan et al., 1995) which is a prerequisite for its degradation and release of NF-kB / rel proteins. The fact that CsA is able to block the activity of ionomycin supports this concept. It is also possible however that calcineurin is upstream of another protein, called bcl-3. In fact TCR signalling is thought to mediate bcl-3 activation (Lenardo and Siebenlist, 1994), although it is not known yet if calcineurin is involved. The role of this molecule is to remove p50 dimers that negatively regulate gene expression in the nucleus. The presence of lower levels of p50 after P/I stimulation supports that. At the same time bcl-3 has the ability to act positively by using its transactivation domain and aiding p52 transcriptional ability. The binding of bcl-3 in the NF-kB DNA complex would also lead to an increase in the weight of the complex, which may explain the shift in the NF-kB complexes observed in the presence of P/I but not after PMA+CD80 stimulation in human T cells. The inability of CD28 signals to activate bcl-3 may therefore not allow strong activation of NF-kB.

Lane	1	2	3	4	5	6	7	8
PMA	-	+	+	+	+	+	+	+
Iono.	-	-	+	+	-	-	-	-
CD80	-	-	-	-	+	+	+	+
CsA	-	-	-	+	-	+	-	-
xs NF-kB	-	-	-	-	-	-	+	-
xs AP1	-	-	-	-	-	-	-	+

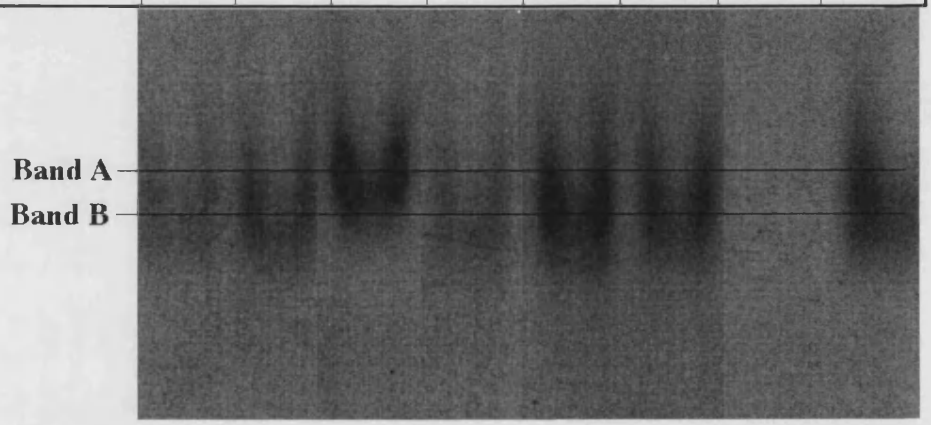

FIGURE 4.18: NF-kB DNA binding activity of human T cells. 10^7 human resting T cells were stimulated as specified above at a concentration of $2x10^6$ cells /ml. PMA was used at 5ng/ml, ionomycin at 1μM and CHO-CD80 cells were used at 1:3 ratio to jurkat T cells. CsA treated cells were pre-incubated for 30 minutes with the inhibitor. Nuclear extracts were obtained after 8 hours and were then incubated with radiolabelled NF-kB oligonucleotide and run in a gel as specified in the materials and methods. Competition assays (lanes 7 and 8) were performed with a 100 fold excess unlabelled oligonucleotide.

4.2.4.4b: AP1 DNA binding activity in human T cells.

The signals initiated by P/I and PMA+CD80 were also investigated in their ability to induce AP1 DNA binding activity in human resting T cells. The results in **figure 4.19** showed that AP1 binding activity was minimal in freshly purified human resting T cells (lane 1). However PMA was able alone to act and substantially induce this transcription factor (lane 2). In fact ionomycin only had a small additional effect in this activation which was blocked by CsA (lanes 3 and 4). CD80 on the other hand was unable to affect the DNA binding ability of AP1 (lanes 5 and 6). Competition studies showed the specificity of the complexes (lanes 7 and 8). Thus, these results further supported the data obtained with jurkat T cells which suggested that CD80 may not affect AP1 DNA binding activity significantly, but mainly participate in the induction of the transcriptional ability of AP1.

4.2.4.4c: NFAT DNA binding activity in human T cells.

The effect on the transcription factor NFAT was also examined **(figure 4.20)**. Although NFAT also contains AP1 proteins the ability of the transcription factor to bind the corresponding NFAT DNA site, requires the cytoplasmic factor (NFATp / NFAT1 or NFATc / NFAT2) (Northrop et al., 1993; Rao, 1997). Thus, the DNA binding activity of this transcription factor will theoretically only be seen if NFAT is translocated to the nucleus, a process that requires a calcium / calcineurin dependent pathway. Thus, not surprisingly, PMA was unable to induce NFAT (lane 2) despite its ability to induce AP1. Furthermore, the only stimulation that was able to induce NFAT DNA binding activity was P/I (lane 3) which provides the necessary signals for the activation of calcineurin and subsequently the dephosphorylation of NFAT and translocation to the nucleus. CsA was able to completely prevent this activation (lane 4). Clearly, these results supported the studies in jurkat T cells and showed that a calcium signal is required for the translocation of NFAT to the nucleus. Additionally, pathways mediated by CD80 do not seem able to override this need, as studies with CD28 antibodies have suggested (Ghosh et al., 1996; Lyakh et al., 1997).

Lane	1	2	3	4	5	6	7	8
PMA	-	+	+	+	+	+	+	+
Iono.	-	-	+	+	-	-	-	-
CD80	-	-	-	-	+	+	+	+
CsA	-	-	-	+	-	+	-	-
xs NF-kB	-	-	-	-	-	-	+	-
xsAP1	-	-	-	-	-	-	-	+

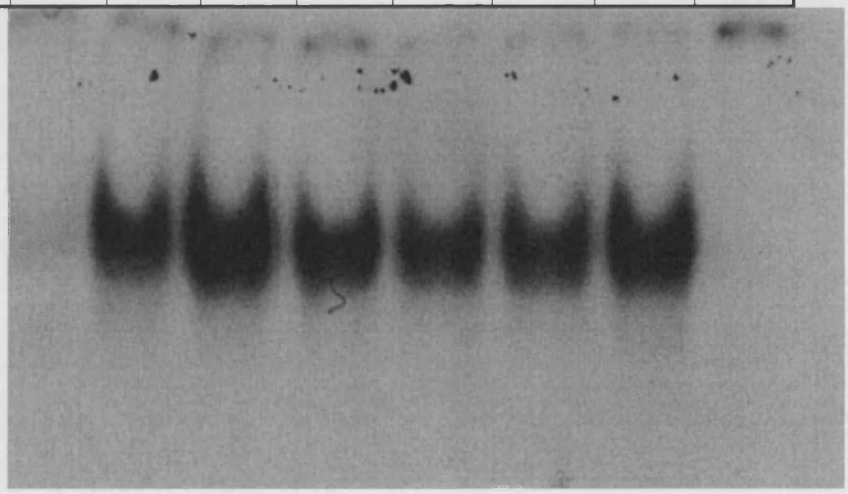

FIGURE 4.19: AP1 DNA binding activity of human T cells. 10^7 human resting T cells were stimulated as specified above at a concentration of $2x10^6$ cells /ml. PMA was used at 5ng/ml, ionomycin at 1μM and CHO-CD80 cells at a ratio of 1:3 to jurkat T cells. CsA treated cells were pre-incubated for 30 minutes with 1μg/ml of the inhibitor. Nuclear extracts were obtained after 8 hours and were then incubated with radiolabelled AP1 oligonucleotide and run in a gel as specified in the materials and methods. Competition assays (lanes 7 and 8) were performed with a 100 fold excess unlabelled oligonucleotide.

Lane	1	2	3	4	5	6
PMA	-	+	+	+	+	+
Iono.	-	-	+	+	-	-
CD80	-	-	-	-	+	+
CsA	-	-	-	+	-	+

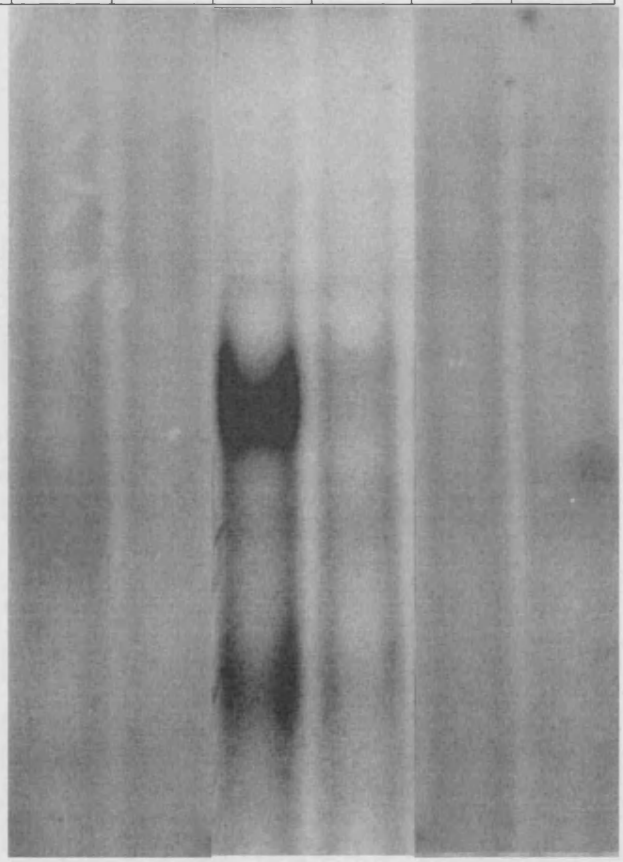

FIGURE 4.20: NFAT DNA binding activity of human T cells. 10^7 human resting T cells were stimulated as specified above at a concentration of $2x10^6$ cells /ml. PMA was used at 5ng/ml, ionomycin at 1μM and CHO-CD80 cells at a ratio of 1:3 jurkat T cells. CsA treated cells were pre-incubated for 30 minutes with 1μg/ml of the inhibitor. Nuclear extracts were obtained after 8 hours and were then incubated with radiolabelled NFAT oligonucleotide and run in a gel as specified in the materials and methods.

4.2.5: An investigation in to the nature of the possible factor(s) that mediate PMA+CD80 induced proliferation.

Since stimulation with PMA+CD80 appeared to be independent of IL-2, the signals induced must either be able to initiate proliferative responses directly in T cells or mediate the production of a soluble factor(s) that can induce proliferation in an autocrine fashion. If the latter is true it is important to try and understand the nature of such a factor. In an attempt to answer these questions the ability of supernatants from cells stimulated with PMA+CD80, to induce proliferation of activated T cell blasts was examined. Additionally mRNA was obtained from cells stimulated with PMA+CD80, which was used for RT-PCR studies in order to establish the presence or absence of certain known cytokine mRNAs that might be induced.

4.2.6.1: Proliferative potential of the supernatants obtained from PMA+CD80 stimulated T cells.

Activated T cell blasts are responsive to a number of cytokines and other soluble factors, because they express a number of appropriate receptors at their surface. In the studies performed here supernatants from human T cells activated with PMA alone, PMA+CD80 or P/I (in the presence or absence of CsA) were examined for their ability to induce proliferation on activated T cells. In order to minimise residual carry over of PMA and / or ionomycin in the supernatants, cells from the original cultures were washed thoroughly after 4 hours of stimulation. This committed the T cells to proliferate but removed any PMA and / or ionomycin within the supernatants taken subsequently at 48 hours after stimulation. The supernatants were then used to stimulate T cell blasts obtained from a culture that resulted from the induction of PBMCs with SEA, as mentioned in chapter 3. Specifically, 4 days after activation cells were utilised after being extensively washed in order to remove any proliferative factor present in the culture and to reduce the basal levels of proliferation in the assays. The ability of IL-2 to induce proliferation was used as a positive control, in order to make sure that the SEA

blasts were able to expand in response to cytokines. When activated T cell blasts were stimulated it was found that the supernatants of T cells simulated with PMA alone did not result to a proliferative response above the basal levels (**figure 4.21**). In contrast a strong proliferative response was seen in blasts treated with supernatant from either PMA+CD80 or P/I treated T cells.

The above results suggested that a soluble factor with a proliferative potential, is actually produced by T cells stimulated with PMA+CD80. However, since human resting T cells can be induced to proliferate in the presence of CsA, this factor must also be induced under these conditions. In other words, supernatants obtained from cells stimulated with PMA+CD80+CsA should be equivalently potent as the ones obtained from cells stimulated without CsA. Additionally the presence of CsA in the responding culture (i.e. the activated T cell blasts) should not prevent the ability of the factor to induce proliferation. As **figure 4.21** shows the presence of CsA within the supernatants of PMA+CD80 stimulated cells did not alter their ability to proliferate T cell blasts. In contrast, the ability of equivalent supernatants obtained from T cells stimulated with P/I+CsA were not able to support proliferation. This was expected since IL-2 production via P/I is highly sensitive to CsA as above data showed. However they also suggest that any possible additional proliferative factors induced by P/I are either also sensitive to CsA, or they are unable to act without IL-2. The effect of CsA on the proliferation of activated T cell blasts directly was also as predicted (**Figure 4.22**) since proliferation stimulated by the supernatants was only modestly inhibited by CsA. The fact that supernatants from P/I stimulated T cells can also act in a CsA resistant fashion is not surprising since IL-2 signalling is not known to require calcium nor calcineurin signals. Supporting that IL-2 induced proliferation of T cell blasts was not affected by CsA in these experiments. Collectively, the results suggest that PMA+CD80 may be able to support proliferation due to the induction of one or more soluble factors that is produced and acts in a CsA resistant fashion.

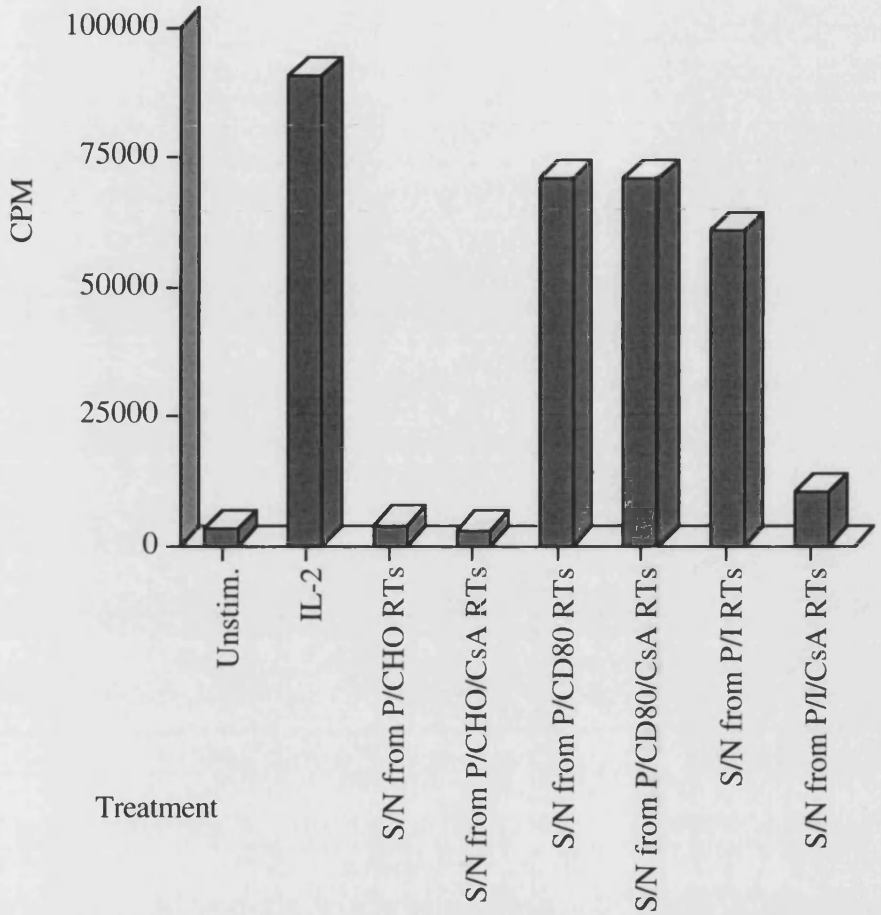

Figure 4.21: Examination on the production of the proliferative factor (s) that is (are) induced by stimulated T cells. Purified human resting T cells were stimulated in the presence or absence of CsA (30 minutes pre-treatment), with 5ng/ml PMA plus CHO cells at a ratio of 1:3 T cells (P/CHO) or CHO-CD80 cells at a ratio of 1:3 T cells (P/CD80) or 1μM ionomycin (P/I). Cells were washed at 4 hours, supernatants were collected at 48 hours and used to stimulate day 4 SEA (10ng/ml) activated T cell blasts. Control blasts were left untreated (unstim.) to determine the basal proliferative potential of these cells, or treated with 100ng/ml IL-2 (IL-2). Proliferation of the blasts was measured at 24 hours, by the incorporation of ^{3}H-thymidine during an additional 18 hour incubation.

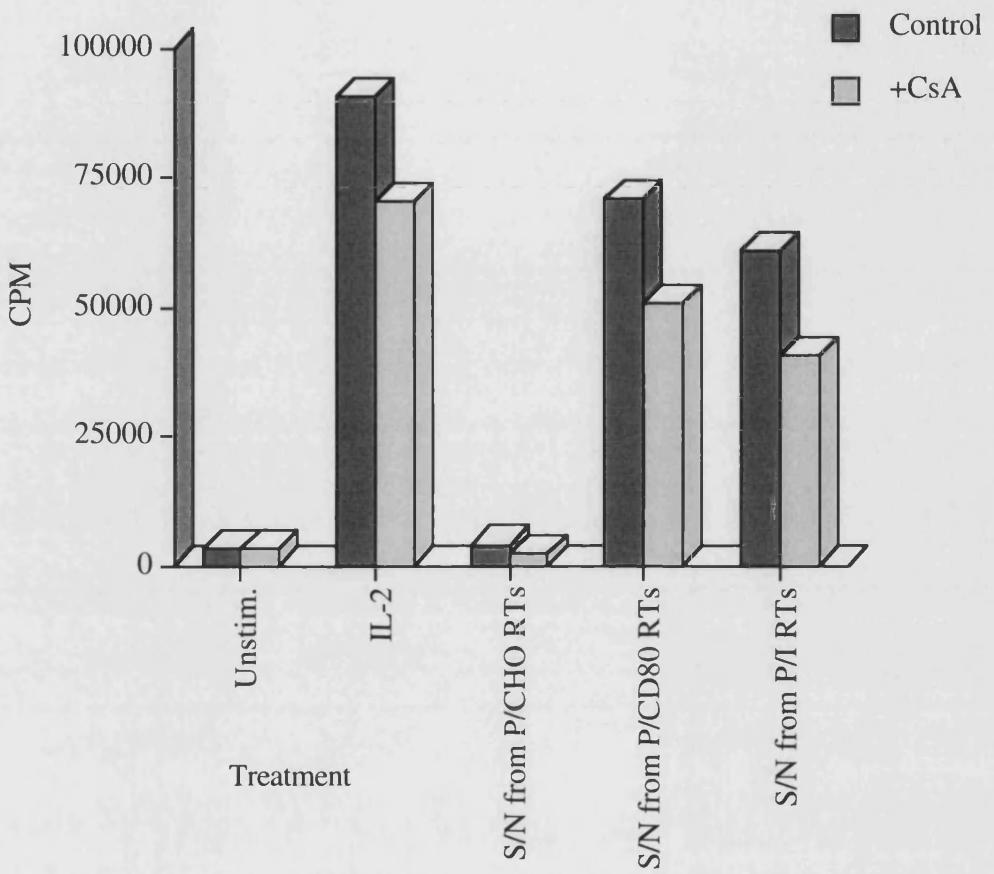

Figure 4.22: Effect of CsA on the proliferative potential of supernatants obtained from PMA+CD80 stimulated T cells. Purified human resting T cells were stimulated with 5ng/ml PMA plus CHO cells at a ratio of 1:3 T cells (P/CHO) or CHO-CD80 cells at a ratio of 1:3 T cells (P/CD80) or 1μM ionomycin (P/I). Cells were washed at 4 hours, supernatants were collected at 48 hours and used to stimulate day 4 SEA (10ng/ml) activated T cell blasts. Control blasts were left untreated (unstim.) to determine the basal proliferative potential of these cells, or treated with 100ng/ml IL-2. The additional effect of CsA was determined by pre-treating cells with the inhibitor for 30 minutes. Proliferation of the blasts was measured at 24 hours, by the incorporation of ^{3}H-thymidine during an additional 18 hour incubation.

4.2.6.2: Examination of the possible role of other cytokine(s) on T cells activated with PMA+CD80.

The above data suggested that PMA+CD80 are able to induce the production of a molecule that can support proliferation of human T cells. Considerable evidence suggests that IL-2 does not perform such a task in this case, although they do not exclude its possible production in small amounts. To further examine IL-2 and the possible involvement of other cytokines in this system, mRNA was purified from T cells stimulated with PMA, P/I or PMA+CD80 (with or without CsA) for 4 hours. Primers specific for IL-2, IL-4, IL-10 and IL-13 were designed from the corresponding cDNAs and used for RT-PCR studies, in order to establish the presence of these cytokines. The levels of IL-2 mRNA are seen in **figure 4.23a**. No IL-2 mRNA was detected on unstimulated or PMA activated T cells (lanes 1 and 2). Stimulation with PMA+CD80 resulted in some levels of IL-2 mRNA that were only partially blocked by CsA (lanes 3 and 4), but only when T cells were stimulated with P/I was IL-2 induced in high levels (lane 5). Considering the fact that control GAPDH levels in these assays were lower after P/I than any of the other stimulations, it is possible that these levels are actually underestimated and that P/I can induce IL-2 mRNA even more. Thus, as the results obtained by the IL-2 detection assays, there was a significant difference between the levels of IL-2 induced by P/I and PMA+CD80. Nevertheless the low levels of IL-2 after PMA+CD80 suggested that CD28 may be affecting later stages of gene expression without affecting transcription. Many studies have examined the levels of IL-2 mRNA in the cell and assumed that any increase results from enhanced transcription. However, resting T cells are not metabolically inactive, but are characterised by a balance between activating and inhibiting pathways. Thus a relative steady / basal level of transcription in resting T cells may allow IL-2 mRNA to be present in the cells. At the same time pathways that degrade mRNAs may be active and keep the levels low and steady. Stimulation with CD28 may therefore prevent the latter without affecting transcription. As a result IL-2 mRNA will be

Lane	1	2	3	4	5	6		M
PMA	-	+	+	+	+	+		-
Iono.	-	-	-	-	+	+		-
CD80	-	-	+	+	-	-		-
CsA	-	-	-	+	-	+		-

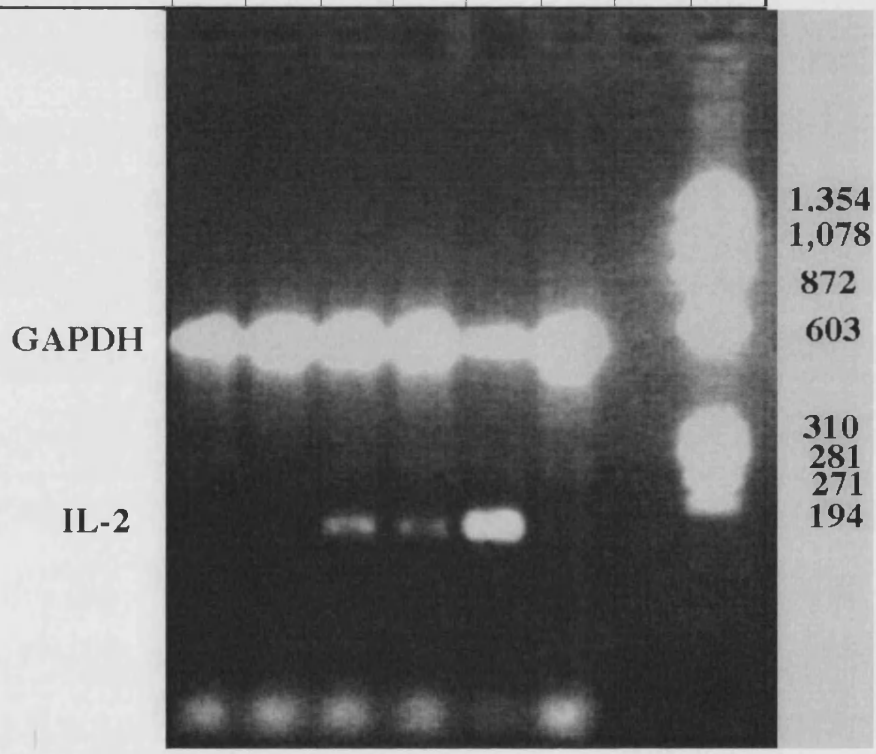

FIGURE 4.23a: Induction of IL-2 mRNA levels on human T cells. 10^7 human resting T cells were stimulated as specified above at a concentration of $2x10^6$ cells /ml. PMA was used at 5ng/ml, ionomycin at 1μM and CHO-CD80 cells at a ratio of 1:3 jurkat T cells. CsA treated cells were pre-incubated for 30 minutes with 1μg/ml of the inhibitor. RNA extraction was performed 8 hours after stimulation and RT-PCRs were run as detailed in materials and methods. Lane M represents the DNA molecular weight markers obtained from ϕX174 DNA digested with *Hae* III. The results are representative of two independent experiments.

more stable, more will be detected and more IL-2 protein will be secreted. In other words, the ability of others and of studies here, to detect low levels of IL-2 after stimulation with PMA+CD80 may be the result of increased mRNA stability, a function that others have associated with the costimulatory properties of CD28 (Lindsten et al., 1989; Umlauf et al., 1995; June et al., 1989). It is therefore possible that CD28 induces certain proteins that are thought to bind AU-rich sequences at mRNAs (Bohjanen et al., 1991) and probably avoid their susceptibility to RNAse that mediate degradation. The levels of IL-4 mRNA are seen in **figure 4.23b**. Clearly, no IL-4 mRNA was detected in unstimulated nor PMA activated T cells (lanes 1 and 2). Although a small increase was observed with PMA+CD80, this was sensitive to CsA (lanes 3 and 4) which therefore makes it an unlikely candidate for the mediator of PMA+CD80 responses. Clearly, the most striking increase of IL-4 mRNA was observed after treatment with P/I in a CsA sensitive fashion (lanes 5 and 6). Similar results were obtained with IL-10 **(figure 4.23c)**. Low levels were this time detected on unstimulated T cells (lane 1) which were not increased by PMA alone (lane 2) but were enhanced slightly with PMA+CD80 (lane 3). Again however, this stimulation was sensitive to CsA (lane 4). The levels of IL-10 mRNA were mainly increased by P/I, but this time in a partially CsA sensitive manner (lane 5 and 6). Clearly, neither IL-4 or IL-10 appeared to be likely products of PMA+CD80 stimulation and are therefore unlikely mediators of the proliferative effects of this stimulation.

In contrast, the results with IL-13 suggested that this cytokine may at least be one of the products of PMA+CD80 activation **(figure 4.23d)**. Basal levels of IL-13 mRNA in unstimulated cells were induced with PMA+CD80 (lane 1-3). More interestingly, the synergistic effect of PMA+CD80 was resistant to CsA (lane 4). P/I stimulation on the other hand resulted in a similar enhancement of IL-13 levels, but in a CsA sensitive fashion (lanes 5 and 6). Thus, IL-13 is suggested to be one of the candidate targets of CD80. However, although this result agrees with others which have shown the ability of CD80 to upregulate IL-13 (Minty et al., 1993), this cytokine is an

Lane	M		1	2	3	4	5	6
PMA	-		-	+	+	+	+	+
Iono.	-		-	-	-	-	+	+
CD80	-		-	-	+	+	-	-
CsA	-		-	-	-	+	-	+

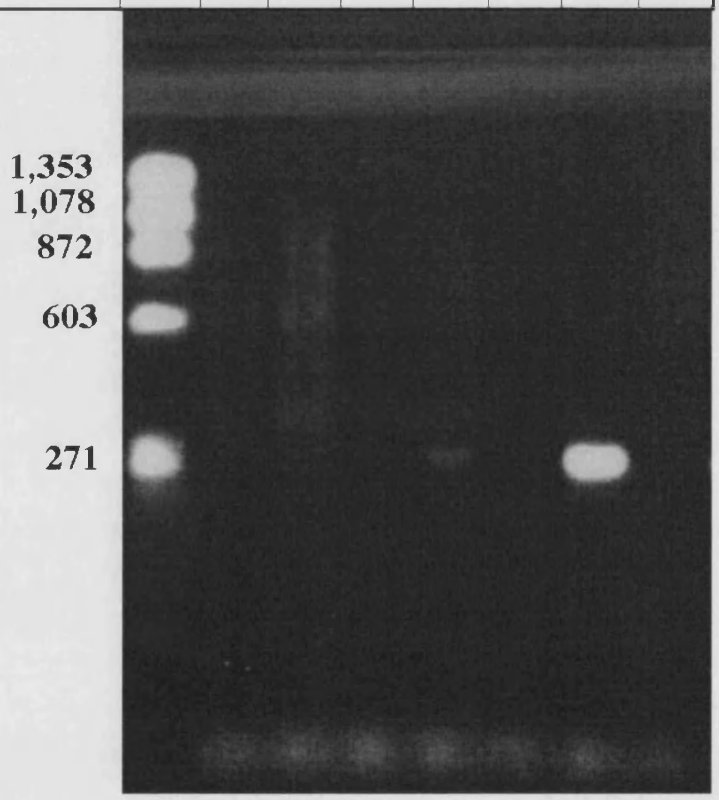

FIGURE 4.23b: Induction of IL-4 mRNA levels on human T cells. 10^7 human resting T cells were stimulated as specified above at a concentration of $2x10^6$ cells /ml. PMA was used at 5ng/ml, ionomycin at 1μM and CHO-CD80 cells at a ratio of 1:3 jurkat T cells. CsA treated cells were pre-incubated for 30 minutes with 1μg/ml of the inhibitor. RNA extraction was performed 8 hours after stimulation and RT-PCRs were run as detailed in materials and methods. Lane M represents the DNA molecular weight markers obtained from ϕX174 DNA digested with *Hae* III. The results are representative of two independent experiments.

Lane	M	1	2	3	4	5	6
PMA	-	-	+	+	+	+	+
Iono.	-	-	-	-	-	+	+
CD80	-	-	-	+	+	-	-
CsA	-	-	-	-	+	-	+

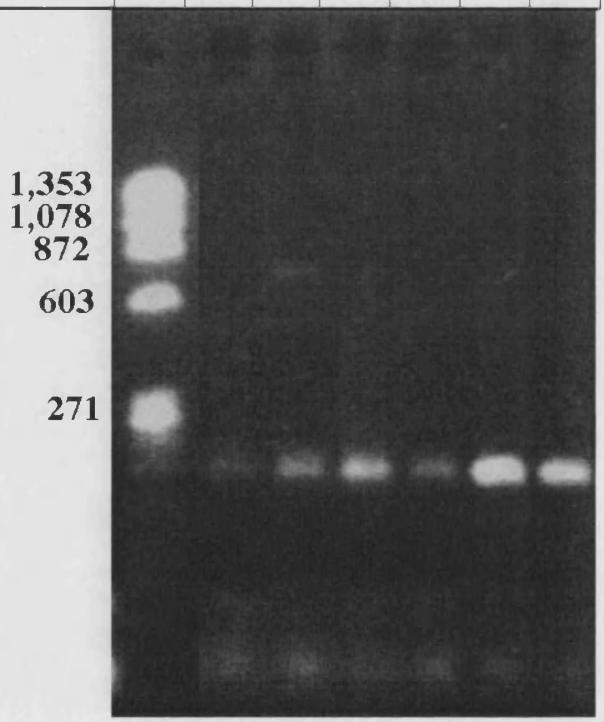

FIGURE 4.23c: Induction of IL-10 mRNA levels on human T cells. 10^7 human resting T cells were stimulated as specified above at a concentration of $2x10^6$ cells /ml. PMA was used at 5ng/ml, ionomycin at 1μM and CHO-CD80 cells at a ratio of 1:3 jurkat T cells. CsA treated cells were pre-incubated for 30 minutes with 1μg/ml of the inhibitor. RNA extraction was performed 8 hours after stimulation and RT-PCRs were run as detailed in materials and methods. Lane M represents the DNA molecular weight markers obtained from ϕX174 DNA digested with *Hae* III. The results are representative of two independent experiments.

Lane	M		1	2	3	4	5	6
PMA	-		-	+	+	+	+	+
Iono.	-		-	-	-	-	+	+
CD80	-		-	-	+	+	-	-
CsA	-		-	-	-	+	-	+

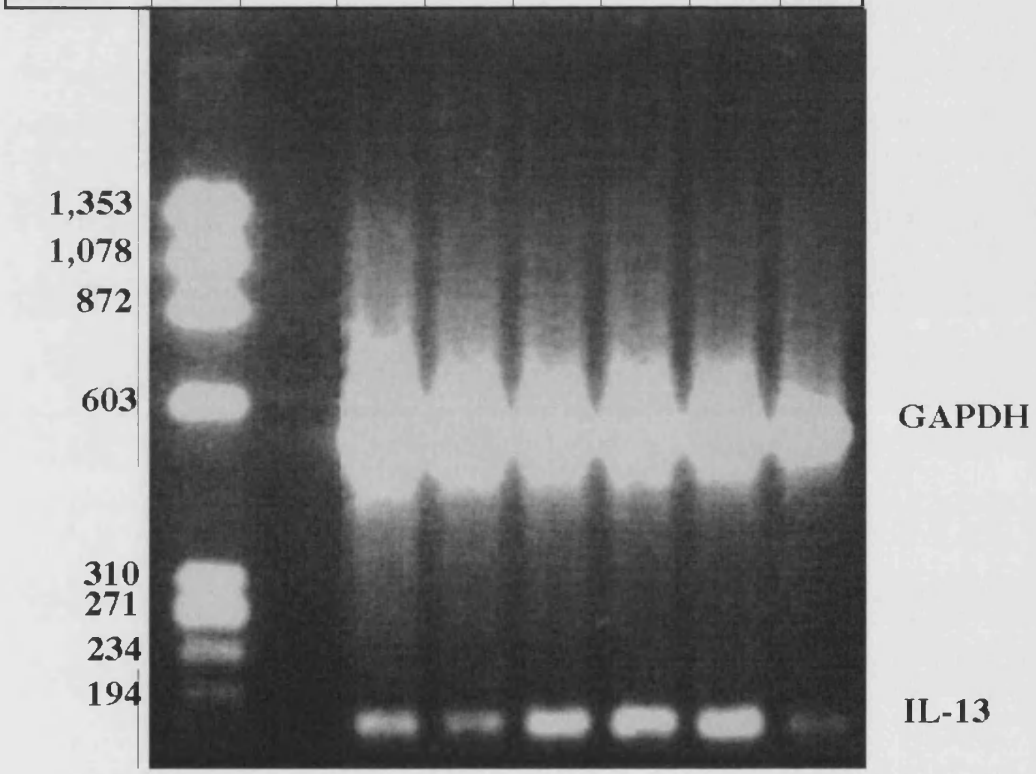

FIGURE 4.23d: Induction of IL-13 mRNA levels on human T cells. 10^7 human resting T cells were stimulated as specified above at a concentration of $2x10^6$ cells /ml. PMA was used at 5ng/ml, ionomycin at 1μM and CHO-CD80 cells at a ratio of 1:3 jurkat T cells. CsA treated cells were pre-incubated for 30 minutes with 1μg/ml of the inhibitor. RNA extraction was performed 8 hours after stimulation and RT-PCRs were run as detailed in materials and methods. Lane M represents the DNA molecular weight markers obtained from ϕX174 DNA digested with *Hae* III. The results are representative of two independent experiments.

unlikely mediator of PMA+CD80 proliferation because it is not suggested to be involved in T cell proliferation, but instead affects B cells (Minty et al., 1993). Thus, these results were not able to clearly establish a cytokine that may be responsible for the proliferative potential of PMA+CD80 stimulated T cells.

4.2.7: IL-2 independent proliferation of activated T cells via CD80.

The data obtained so far have suggested that costimulation of PMA treated T cells is resistant to CsA and can be independent of IL-2. Interestingly, previous work in our laboratory has shown that CD80 can act in a similar fashion in activated T cells and further enhance T cell activation without IL-2 production (Edmead et al., 1996). Additionally, similar to the stimulation of PMA+CD80 on resting T cells, CD80 alone on activated T cells increased the levels of NF-kB and AP1 in the nucleus of the T cells, but did not induce NFAT translocation (Edmead et al., 1996). Thus, again CD80 appears to perform its proliferative function independently of IL-2. In order to further examine this, IL-2 receptor blocking experiments were performed. Interestingly, the results suggested that not all the proliferative effect of CD80 on activated T cells blasts is IL-2 independent (**figure 4.24**). Thus, day 4 SEA activated T cells were further induced approximately 5-fold by CD80 in terms of proliferation and although the addition of anti-IL2Rα prevented this proliferation, a 3-fold induction remained intact, suggesting that not all CD80 effects in this system are mediated via IL-2. Additional experiments were performed with CsA in order to examine the role of calcineurin in this stimulation. Interestingly, CsA was able to partially reduce proliferation to levels equal to the ones left intact with the anti-IL2Rα antibody (**figure 4.24**). These results suggest that CD80 can induced proliferation of activated T cells in an IL-2 dependent manner, by synergising with calcium / calcineurin dependent signals. At the same time however, CsA resistant signals of CD80 can further enhance proliferation of activated T cells without the help of IL-2. Thus, these data further support the ability of CD80 to proliferate T cells independently of IL-2.

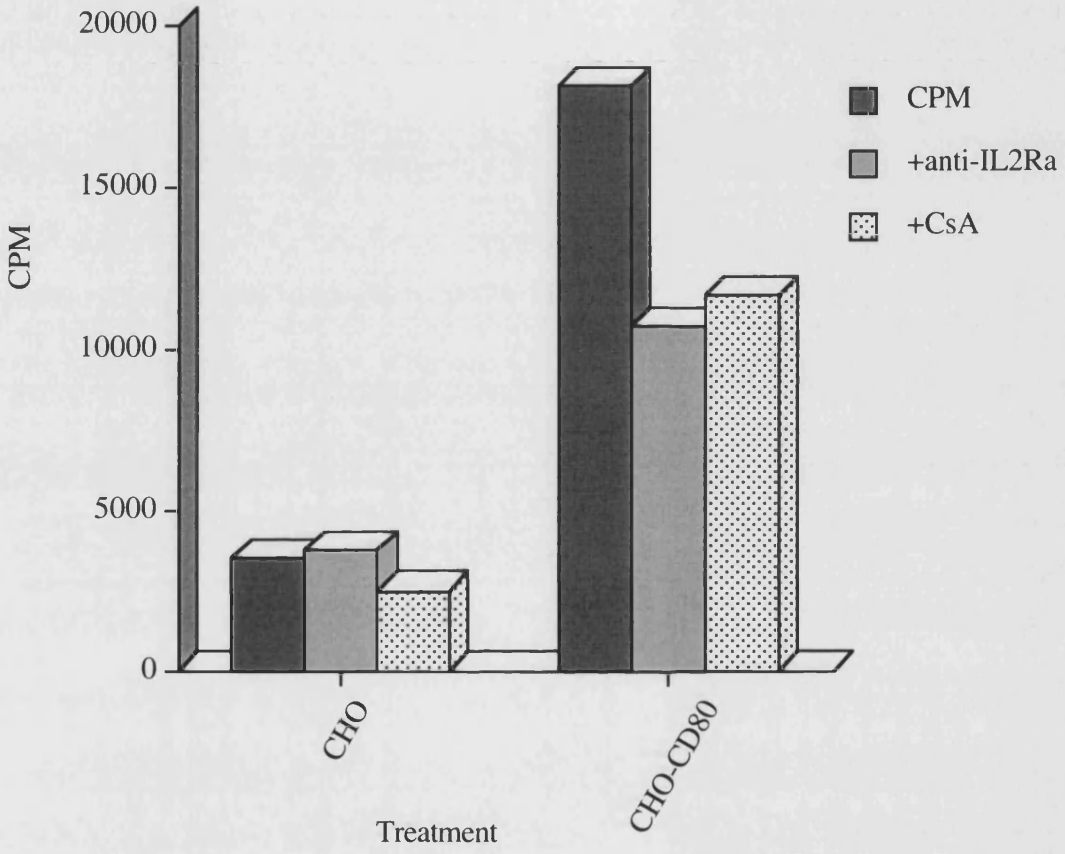

Figure 4.24: Effect of blockade of the IL-2 receptor and of CsA on the CD80 mediated proliferation of activated T cell blasts. Day 4 SEA (10ng/ml) activated T cell blasts were left alone or pre-treated with 10μg/ml anti-IL-2 Rα antibody or 1μg/ml CsA and stimulated with CHO cells or CHO-CD80 cells (both a ratio of 1:3 T cells). Proliferation of the blasts was measured at 24 hours, by the incorporation of ^{3}H-thymidine during an additional 18 hour incubation.

4.3: DISCUSSION

One of the most obvious features associated with CD28 costimulation is the induction and secretion of a number of cytokines, of which IL-2 has been the best studied (June et al., 1989; Linsley et al., 1991a; Fraser et al., 1992; Nunes et al., 1993; Kuiper et al., 1994). Specifically CsA resistant signals downstream of CD28 are thought to be involved in the activation of a number of transcription factors including NF-kB (Bryan et al., 1994; Lai and Tan, 1994; Edmead et al., 1996; Herhaj and Sun, 1998; Civil et al., 1996; Harhaj et al., 1996), AP1 (Granelli-Piperno and Nolan, 1991; McGuire and Iacobelli, 1997; Edmead et al., 1996), NFAT (Ghosh et al., 1996; Lyakh et al., 1997) and a specific CD28 response complex which binds to the IL-2 promoter (Verweij et al., 1991; Ghosh et al., 1993; Lai et al., 1995). In addition CD28 is thought to act at the post-transcriptional level by increasing the stability of the produced mRNA transcripts (Lindsten et al., 1989; Umlauf et al., 1995). Consequently, CD28 costimulation and IL-2 production have been considered synonymous by a number of studies (Linsley et al., 1991a; Fraser et al., 1992; Nunes et al., 1993; Kuiper et al., 1994; Seder et al., 1994). However, the results presented in chapter 3 clearly showed that the extent of IL-2 production does not always correlate with CD28 costimulation or the strength of the corresponding proliferative response. The results presented in this chapter go even further and indicate that the ability of CD28 to synergise with the phorbol ester PMA and induce ^{3}H-thymidine uptake by purified resting T cells, is resistant to CsA and independent of IL-2. Instead, a different soluble proliferative factor seems to be utilised under these conditions, suggesting that IL-2 is not the only factor that can mediate proliferation of resting T cells after CD28 costimulation. This is also supported by studies with T cells from IL-2$^{-/-}$ mice which are still able to respond to CD3+CD28 stimulation, although at lower levels than control mice (Razi-Wolf et al., 1996). Thus, IL-2 may not always be a vital mediator of the proliferative responses that CD28 induces.

4.3.1: Defective IL-2 transcription by PMA+CD80.

The low levels of IL-2 produced by PMA+CD80 was the first indication that IL-2 is not vital under this type of stimulation. Even more striking however was the inability of PMA+CD80 to induce the full repertoire of the transcription factors that participate in the activation of the IL-2 gene. Specifically, the results presented here show that CD80 can act with PMA in a CsA resistant fashion and participate in the activation of NF-kB and AP1 transcription factors that regulate IL-2 gene expression, but not NFAT. Both in human T cells and in jurkat cells CD28 could synergise with PMA and increase the amount of NF-kB / rel proteins that are translocated in the nucleus and therefore form a complex in the promoter of the IL-2 gene. However, the NF-kB complexes induced by PMA+CD80 were not fully active when compared to the ones resulting by P/I. This is supported by the luciferase studies performed here, but also by the supershift assays that suggested the presence of a higher amount of the inactive rel protein p50 in the NF-kB DNA complexes that PMA+CD80 induce. Clearly, CD28 induces NF-kB by mainly promoting its translocation in the nucleus. This is in agreement with a number of previous reports that have suggested that CD28 can increase and prolong NF-kB translocation (Bryan et al., 1994; Lai and Tan, 1994; Edmead et al., 1996; Harhaj et al., 1996), but that a calcium signal is needed for high transactivation (Kanno and Siebenlist, 1996; Lai and Tan, 1994; Frantz et al., 1994; Steffan et al., 1995; Shatrov et al., 1997). Contrary to NF-kB, the effect of CD28 on AP1 seems to be concentrated on activation of transcriptional activity. As the results showed, CD80 has the ability to further enhance transcriptional activity induced by PMA in a CsA resistant fashion. This ability of CD28 to potentiate AP1 induction is stronger when CD3 is used as primary signal. Clearly, the effect of CD28 must be concentrated on the activation of proteins that are already present in the nucleus. In this respect CD28 is thought to play a vital role in the strong activation of JNK, the kinase that is responsible for the phosphorylation and activation of c-jun, an important transcription factor for the assembly of active AP1 (Hibi et al., 1993; Derijard et al., 1994; Minden et al.,

1995). Interestingly JNK has also been suggested to act upstream of NF-kB as well (Malinin et al., 1997; Karin and Delhase, 1998; Meyer et al., 1996). Other enzymes downstream of CD28, that participate in the activation of NF-kB are still unclear. A number of IkB kinases are being discovered (DiDonato et al., 1997; Cao et al., 1996) and have recently been suggested to act downstream of CD28 as well (Herhaj and Sun, 1998). However, rapamycin has been reported to prevent the ability of CD28 to induce NF-kB by inhibiting the degradation of IkBα (Lai and Tan, 1994). It is unclear however if this is because of the inhibition of p70S6 kinase or other enzymes downstream of the target of rapamycin (TOR). PKCζ has also been suggested to activate NF-kB (Muller et al., 1995; Lozano et al., 1994), but its role downstream of CD28 has not been verified.

Despite the ability of CD28 to participate in the activation of NF-kB and AP1, the results presented here were unable to detect an ability of CD80 to induce NFAT, which requires a calcium / calcineurin dependent signal. P/I stimulation was able to induce NFAT activity, whereas CD3 was able to activate NFAT alone with an additional effect by CD80 probably due to the ability of CD80 to enhance the activation of the AP1 proteins of NFAT. The fact that a similar enhancement is not seen on the activation of NFAT by P/I is probably due to the already strong effect of PMA on AP1, compared to CD3. It therefore seems that the participation of CD28 on the activation of IL-2 lies mainly in the induction of NF-kB and AP1, with NFAT being a target of the T cell receptor. As a result, the signals induced by PMA+CD80 were found incapable of promoting IL-2 transcription unless ionomycin or another calcium elevating stimulus (e.g. CD3) was also used to induce NFAT. This contrasts other reports that have suggested a partially calcineurin independent mechanism to be initiated by CD28 (Ghosh et al., 1996; Lyakh et al., 1997; Nebl et al., 1998). Importantly however, the stimulation of NFAT observed in these reports was limited compared to that induced by other stimuli (e.g. P/I). Furthermore, as in most of costimulatory studies, antibodies were used to stimulate the CD28 receptor, which

may initiate certain signals that are not physiologic and do not represent the effects of the natural ligand CD80 (Nunes et al., 1994).

The ability of CD80 to induce low levels of IL-2 and mediate proliferative responses independently of IL-2, seem to initially contradict other studies that have suggested that PMA and anti-CD28 antibodies can stimulate T cells and promote the production of the IL-2 cytokine both at the mRNA levels (June et al., 1987; Linsley et al., 1991a; Thompson et al., 1993; June et al., 1989) and the protein levels (Ghosh et al., 1996; Seder et al., 1994). The data shown in **figure 4.2** of this chapter however, suggest that this may be an effect that cross-linked anti-CD28 antibodies can mainly confer. A large increase of IL-2 production is seen in T cells stimulated with PMA and cross-linked anti-CD28 antibodies, which is however largely blocked by CsA suggesting that a calcium / calcineurin pathway is initiated under these conditions. This is not surprising since CD28 antibodies are suggested to induce different and more widespread signals than CD80 and / or CD86 (Nunes et al., 1994) and amongst these differences, CD28 antibodies are able to increase calcium levels in the cell when cross-linked (Ohnishi et al., 1995; Nunes et al., 1993; Ledbetter et al., 1992; Ohnishi et al., 1995). As a result calcineurin may be activated by CD28 antibodies and participate in the production of IL-2. In contrast however, previous reports have suggested that the effect of anti-CD28 antibodies is largely CsA resistant (June et al., 1987; Osorio et al., 1998; Hess and Bright, 1991; June et al., 1989; Lu et al., 1995). In this respect, some levels of IL-2 are left intact in the presence of CsA when cross-linked anti-CD28 antibodies are used. Furthermore, these levels are similar to the ones induced by soluble anti-CD28 antibodies that act in a mostly CsA resistant fashion. It seems therefore that CD28 antibodies are able to initiate signals that are able to induce IL-2 production, but the extent of the CsA resistance may depend on the type of presentation and the extent of cross-linking of the anti-CD28 antibodies. In support of this CsA resistance is only partial in some of the studies that have utilised CD28 antibodies (Hess and Bright, 1991; Thompson et al., 1993). Furthermore soluble CD28 antibodies have not been found able to

produce IL-2 with PMA by others (Ohnishi et al., 1995; Sagerstrom et al., 1993). Even more strikingly and in agreement with the data presented here, the PKC activator bryostatin has been found able to mediate IL-2 production when acting with cross-linked anti-CD28 antibodies, but not when acting with the natural ligand CD80 (Nunes et al., 1994).

Clearly therefore CD80 and soluble (uncross-linked) anti-CD28 antibodies can induce low levels of IL-2 by by-passing the need for calcium and calcineurin. Thus, despite the absence of NFAT activation, low levels of IL-2 are still secreted and detected in the supernatants, when PMA synergises with CD80. This suggested that CD28 may be mediating its effects by enhancing IL-2 production at a post-transcriptional level. Interestingly, although most studies take for granted the ability of CD28 to activate IL-2 in a CsA resistant fashion, only a few have shown the ability of PMA to synergise with CD28 and induce IL-2 transcriptionally in the presence of CsA and even then, the levels induced were low compare to the ones initiated by P/I (Ghosh et al., 1996; Nebl et al., 1998). Supporting a post-transcriptional role of CD28, PMA+CD80 was able to enhance the levels of IL-2 mRNA in the cells in a CsA resistant fashion. Although this level was much lower than the one induced by P/I, it suggested that CD28 may be enhancing the stability of mRNA transcripts in the cells as others have shown (Lindsten at al., 1989; Umlauf et al., 1995; June et al., 1989). Thus even when CD28 does not affect transcription of the IL-2 gene, it is capable of stabilising any mRNA present at unstimulated cells and as a result increase its levels in the cell. Collectively, these results showed that PMA+CD80 mediated proliferation is accompanied by low levels IL-2 production, possibly induced by an increasing stability of IL-2 mRNA and not by an increased transcriptional rate of the IL-2 promoter. Additionally, this activity is resistant to CsA as other studies have shown in the past (June et al., 1987; June et al., 1989; Osorio et al., 1998; Hess and Bright, 1991). Differences in the extent of IL-2 induction observed in different studies may simply be the result of variable sensitivities of the assays used. It must be noted however that high

sensitivity is not always favourable in interpreting such results because it may lead to the saturation of strong signals and at the same time allow weaker signals to be over presented.

Previous work in the laboratory has showed that activated T cell blasts can be further induced to proliferate in the presence of CD80. Interestingly these cells did not produce any detectable levels of IL-2 and were unable to translocate NFAT to the nucleus. In contrast the transcription factors NF-kB and AP1 were activated by CD80 in these cells (Edmead et al., 1996). Thus, these results and the ones presented here do not contradict the importance of CD28 on IL-2 production and the transcription factors involved. The lack of a strong and possibly sustained calcium elevation and the absence of NFAT activation in both cases however, renders CD28 induced signals insufficient. In fact, the results showed here suggested that when a calcium signal is given to the cells aiding NFAT translocation to the nucleus (i.e. P/I or CD3), CD80 can further enhance IL-2 induction.

4.3.2: IL-2 independence of CD28 costimulation

Despite the low levels of IL-2 produced by PMA+CD80, experiments performed here suggested that they are insignificant in terms of proliferative potential. Specifically, blockade of the IL-2 receptor did not prevent the observed proliferation induced by PMA+CD80. In contrast, stimulation with CD3+CD80 was largely blocked by the anti-IL-2 receptor antibody, clearly showing the need for IL-2 under these conditions. Interestingly according to the results presented in chapter 3, stimulation of T cells with CD3+CD80 (but not PMA+CD80) was also blocked by rapamycin that is thought to affect IL-2 signalling. This further supports the concept that PMA+CD80 does not rely on IL-2 signalling for its proliferative potential. One important question that arises from these experiments is why CD80 is able to activate T cells without IL-2 when PMA is utilised but requires IL-2 to costimulate CD3 activated cells. A first possibility is that PMA is able to induce certain signals

that IL-2 would normally activate. In this respect both IL-2 and PMA are suggested to participate in the activation of c-fos and c-jun (Miyazaki et al., 1995; Hibi et al., 1993). However, the ability of PMA to substitute IL-2 signalling is dismissed from the fact that supernatants of PMA+CD80 activated T cells that have been thoroughly washed from PMA, are able to sustain proliferation of T cell blasts **(see figure 4.21 and 4.22)**. Thus, the ability of PMA+CD80 to induce other unknown signals may actually aid this IL-2 independence. Clearly, this factor is not due to an effect that CD28 confers alone since when it acts with anti-CD3 antibodies it largely requires IL-2. Similarly, the observed independence on IL-2 is not an artefact that PMA is able to confer alone, since supernatants of PMA treated T cells were incapable of supporting proliferation. Thus PMA (but not CD3) may be able to induce certain signals that can synergise with CD80 and mediate proliferation either directly or indirectly by promoting the production of a secondary factor. Since supernatants of PMA+CD80 treated resting T cells can cause proliferation on activated T cells, a soluble factor must be able to mediate proliferation. This however does not rule out the presence of additional intrinsic signals induced by PMA+CD80 that are able to mediate or support proliferation. Furthermore, such signals may promote survival of cells and therefore indirectly support or maintain proliferation. Although no such data are presented here, the ability of CD28 to increase bcl-X_L and aid cell survival has been suggested by others (Noel et al., 1996; Sperling et al., 1996; Boise et al., 1995b; Levine et al., 1997; Collette et al., 1997).

The experiments performed here are not conclusive about the nature of the proliferative factor that may mediate proliferation of T cells after PMA+CD80 stimulation. One question arising is whether this cytokine is of Th1 or Th2 type. Certain evidence suggest that the latter may be the case. Firstly, the absence of IL-2 may influence T cell differentiation towards a Th2 type. Supporting this, IL-$2^{-/-}$ cells in mice do not differentiate into IFNγ producing (i.e. Th1) cells (Khoruts et al., 1998). Secondly, studies with NFAT$1^{-/-}$ mice have suggested that NFAT1 may promote the induction of the Th1 type phenotype by negatively regulating the

transcription of the IL-4 gene (Kiani et al., 1997; Xanthoudakis et al., 1996). It must be noted however that, this does not seem to be a universal function of NFAT since similar studies with NFAT2 have actually concluded the opposite and suggests that this type of NFAT transcription factor may promote Th2 cell differentiation (Ranger et al., 1998; Yoshida et al., 1998). Interestingly however, NFAT1 (and not NFAT2) is thought to be the main NFAT transcription factor found in human resting T cells (Rao et al., 1997; Jain et al., 1995; Lyakh et al., 1997; Amasaki et al., 1998). Thus, the absence of NFAT1 induction after PMA+CD80 stimulation may avoid the negative regulation of the IL-4 gene and therefore promote Th2 cytokine production. Thirdly, some similarities between the cells stimulated by PMA+CD80 and Th2 cells also suggest a role for Th2 cytokines. For example, Th2 cells are thought to be activated in a manner less dependent on calcium than Th1 cells (Sloan-Lancaster et al., 1997). However, this mostly refers to established Th2 T cell clones and not cells maturing towards a Th2 phenotype as PMA+CD80 cells may be doing. Finally, both Th2 cells (Lederer et al., 1996a) and cells stimulated with PMA+CD80 (see above) have reduced levels of NF-kB activation. Collectively, these observations suggest a possible role for Th2 cytokines in the stimulatory responses of PMA+CD80 treated T cells. Despite all these indications, the PCR studies performed here did not suggest that the Th2 cytokines IL-4 and IL-10 are suitable candidates since the low levels of induction observed by PMA+CD80 are sensitive to CsA. On the other hand although IL-13 is induced by PMA+CD80, it is not suggested to target and promote T cells proliferation (Minty et al., 1993). Although a more thorough investigation of other known proliferative factors must take place, evidence obtained by other studies suggest that γ chain cytokines can not account for the CD28 dependent proliferation of $IL2^{-/-}$ T cells (Razi-Wolf et al., 1996). Additionally, the data presented here suggest that the gene expression of the responsible factor is not dependent on the transcription factor NFAT (most possibly NFAT1), since PMA+CD80 were unable to activate this transcription factor. Interestingly, these caveats together rule out most known cytokines and other proliferative factors. Thus, the CD28 may be inducing other unknown factors. A clue about this factor

may be present in the fact that although antigenic stimuli on $CD4^{+ve}$ cells of IL-$2^{-/-}$ mice were able to enhance T cell responses *in vivo,* corresponding responses *in vitro*, were not as strong even when endogenous APCs were used (Khoruts et al., 1998). This suggests that a signal driven by non T cells, not utilised in the latter *in vitro* assays, may be able to aid the production of the proliferative factor by T cells. Alternatively, a factor that is produced by certain non T cells may actually be responsible for the proliferation of the T cells directly. Although in the assays performed here the utilisation of pure T cells suggests that the proliferative factor must be made by T cells and act in an autocrine fashion on T cells, the presence of PMA in the stimulation protocol may cause this artefact and avoid the need of the other *in vivo* regulator.

It must be noted that the results presented here not only suggest that IL-2 is not utilised under certain circumstances, but that it can even act as a negative regulator of T cell activation. In both stimulations of PMA+CD80 and P/I, blocking IL2Rα actually resulted in an increase of the proliferative responses. This is rather surprising considering the fact that IL-2 is considered to be the main proliferative factor of T cells (Shibuya et al., 1992; Taniguchi and Minami, 1993; Miyazaki et al., 1995). However this negative potential of IL-2 is also supported by the inflammatory phenotype of IL-$2^{-/-}$ mice (Sadlack et al., 1993) and the ability of IL-$2^{-/-}$ T cells to respond better to antigen in *vivo* (Khoruts et al., 1998; Razi-Wolf et al., 1996). Furthermore, studies with $CD4^{+ve}$ T cells from γ chain deficient mice have suggested that the cells expand *in vivo* due to the lack of a negative regulatory ability of the γ chain cytokines (including IL-2) (Nakajima et al., 1997). Despite this, it is unclear how IL-2 could act both positively and negatively in T cell activation. An attractive possibility is that IL-2 may participate in the induction of CTLA-4 expression on the surface of the T cells after activation (Alegre et al., 1996; Finn et al., 1997; Alegre et al., 1997), a receptor that is thought to antagonise CD28 costimulation and therefore negatively regulate activation. In fact, the autoimmune character of IL-$2^{-/-}$ mice has been suggested to be partly due to the inability of the

cells to induce CTLA-4 on their surface (Alegre et al., 1997). Alternatively IL-2 may sensitise cells to Fas mediated cell death and therefore restrict the extent of the immune response by aiding the death of activated T cells (Lenardo, 1991; Levine et al., 1997). In this respect it is interesting that the anti-apoptotic protein bcl-2 is suggested to sequester calcineurin and prevent the translocation of NFAT4 (Shibasaki et al., 1997) (and possibly other NFAT proteins) in the nucleus, suggesting that the inactivation of NFAT (and possibly IL-2 gene expression) may be one of its anti-apoptotic functions.

Overall the results presented in this chapter showed clearly that CD28 costimulation is not always synonymous with IL-2 production. Other factors that are also induced by CD28 may perform a similar role under certain conditions. Although these experiments have mainly utilised the phorbol ester PMA as an inducer of at least part of the TCR signals, the ability of CD28 to act independently of IL-2 has also been seen in IL-$2^{-/-}$ mice (Khoruts et al., 1998; Razi-Wolf et al., 1996) and also in human T cells activated with CD3 and activated B cells as APCs (Boussiotis et al., 1993). Furthermore the ability of CD28 to support T cell proliferation is also seen here under more physiologic condition by the activation of activated T cells with CD80 alone. Clearly, the importance of IL-2 as a proliferative factor of CD28 signals is not universal and other downstream targets of CD28 may be able to substitute and even act more efficiently than IL-2 in some cases. Again the primary signal that is used to stimulate the cells may play a crucial role, but it is more possible that other factors induced by the APCs or other cells may also determine the role of IL-2 in an immune response.

CHAPTER 5

NEGATIVE REGULATION OF HUMAN T CELL ACTIVATION MEDIATED BY CD80

5.1: INTRODUCTION

The ability of CD80 to act as a costimulator by binding CD28, has until recently been the only directly proven function of this ligand. However, CTLA-4, which is thought to negatively regulate T cell activation (Walunas et al., 1996a; Krummel and Allison, 1996; Krummel and Allison, 1995; Walunas et al., 1994) is a second well established receptor for CD80. Despite that, the ability of this receptor to act negatively has only been shown via the use of CTLA-4 antibodies and not CD80 and has only indirectly been suggested to be mediated by CD80 and / or CD86 in $CD28^{-/-}$ mice (Fallarino et al., 1998; Lin et al., 1998). Interestingly, although CTLA-4 binds CD80 with a considerably greater affinity than CD28 (van der Merwe et al., 1997; Greenfield et al., 1997; Linsley et al., 1994) current evidence suggests that cells transfected with CD80 ligand effectively costimulate responses via CD28 and provide little evidence for ligand operated CTLA-4 function (Edmead et al., 1996; Sansom et al., 1993; Linsley et al., 1991a). This may be explained in part by the substantial differences in the expression levels of CD28 and CTLA-4. In this respect, whereas CD28 is found on resting T cells with relatively abundant surface expression (Sfikakis et al., 1995; Gross et al., 1992), CTLA-4 is undetectable on resting T cells and although it reaches the cell surface only hours after activation, the levels are low at this point (Linsley et al., 1992a; Alegre et al., 1996; Leung et al., 1995). Thus, these data suggest that the cells have to be activated in order for CTLA-4 to appear at the cell surface, act and downregulate responses. Since many forms of T cell stimulation require CD28 engagement for proliferation, the negative effects mediated by CD80 and CD86 will be hidden within their overall costimulatory potential. Although this problem is overcome by antibody studies, it has hampered the study of negative regulation via CTLA-4 by its natural ligands CD80 / CD86. However, research with the natural ligands is vital for the full understanding of the CTLA-4 receptor and specifically its functional competition with CD28.

Certain results, obtained during the studies mentioned in the previous chapters, indicated the ability of CD80 to negatively regulate activation. More profoundly, CD80 was found at certain occasions to render stimulation of T cells with PMA and ionomycin (P/I) less potent. Since CD80 is only known to act via CD28 and / or CTLA-4, these observations may have resulted from the ability of the ligand to mediate negative regulatory functions via CTLA-4. In the results presented in this chapter, this system is utilised and examined further for possible CTLA-4 activity. Additionally, the factors that may control the ability of CD80 to negatively regulate activation by ligating CTLA-4 are examined.

5.2: RESULTS

5.2.1: An examination of the ability of CD80 to negatively regulate T cell activation induced by PMA and Ionomycin (P/I).

The ability of P/I to stimulate T cells independently of CD28 was clearly established in chapter 3. However when PMA was used at high concentrations (5 or 50 ng/ml), the additional presence of CD80 was seen to negatively regulate the responses instead of further activating them. Thus, as also shown here, individually PMA or ionomycin were unable to activate T cells **(Figure 5.1a)** and although both stimuli together (P/I) induced T cell proliferation, the simultaneous presence of CHO-CD80 cells, resulted in a substantial negative effect on T cell proliferation. As expected, PMA alone could effectively synergise with CHO-CD80 cells and costimulate T cell proliferation, thereby confirming the costimulatory potential of the CD80 transfectants on the same cells. These experiments therefore demonstrated that the CD80 ligand could potently costimulate T cells in the presence of PMA and yet was

inhibitory in the additional presence of ionomycin. The levels of IL-2 production after these stimuli are also shown **(Figure 5.1b)**. Interestingly no correlation with the proliferative changes was seen. Stimulations with P/I (with or without CD80) seemed to induce similar levels of the cytokine. Clearly, these results further supported the concept that proliferation and IL-2 are not synonymous, as argued in the last two chapters.

During these assays a slight decrease in proliferation was occasionally observed when using control CHO cells. This was minimal compared to the one induced by CHO-CD80 cells and possibly resulted from interference in T cell clustering by the CHO cells. However, to confirm the specificity of CD80 down regulation, titration experiments with different numbers of CHO-CD80 cells were performed and compared to CHO controls **(Figure 5.2a)**. The extent of downregulation was clearly dependent on the number of CHO-CD80 cells, as the presence of a decreased ratio of transfectants to T cells in culture, increased P/I+CD80 responses. The specificity of the downregulatory effect was also seen by the studies using an anti-CD80 antibody (BB1) to block the CD80 receptor **(Figure 5.2b)**. Increasing doses of BB1 antibody and therefore more potent blockade of CD80 also resulted in increased P/I+CD80 responses. These results clearly show that the downregulation of P/I responses was dependent to the amount of CD80 receptor that was available to the T cells and supported the initial findings that P/I stimulation is substantially, although not completely, inhibited by CD80 in a dose dependent manner.

5.2.1.1: Roles of CD28 and CTLA-4 on P/I+CD80 responses

5.2.1.1a: Signalling via CD28 and / or CTLA-4 during P/I+CD80 responses

As discussed in the introduction, CD80 is able to bind both CD28 and CTLA-4, but whereas the former costimulates T cell responses, CTLA-4 negatively regulates activation. The results above therefore suggested that CD80 may possibly be downregulating T cell responses by binding CTLA-4. It was possible however that CD80 was still engaging CD28 and as a result inducing more rapid responses than

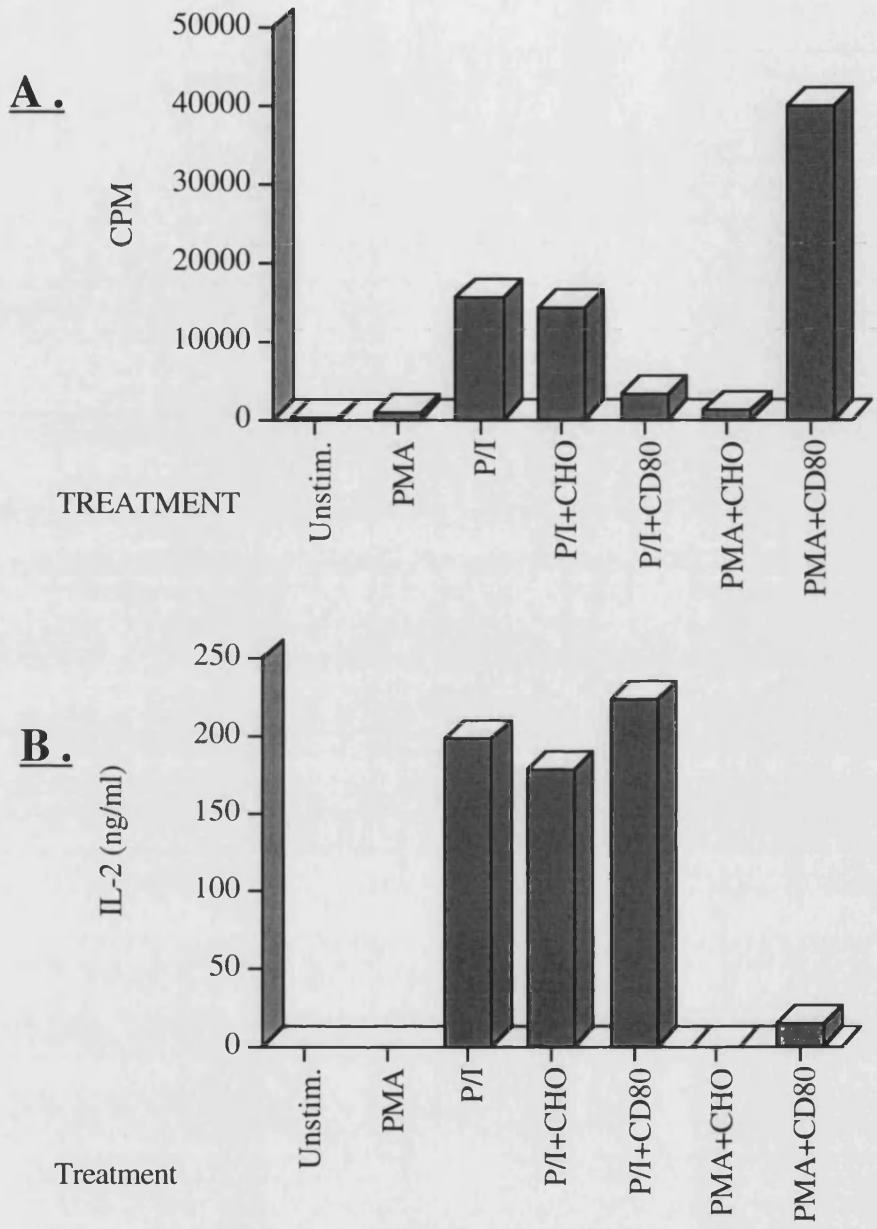

FIGURE 5.1: Negative and positive regulation of T cell activation by CD80. Purified human resting T cells were left unstimulated or treated with 5ng/ml PMA (PMA) or 5ng/ml PMA and 1μM ionomycin (P/I), alone or with the additional presence of CHO or CHO-CD80 cells (both at a ratio of 1:3 T cells). Proliferation **(panel A)** was measured at 72 hours by the incorporation of ^{3}H-thymidine during an additional 18 hour incubation. IL-2 levels **(panel B)** were also determined at 72 hours via ELISA as described in the materials and methods.

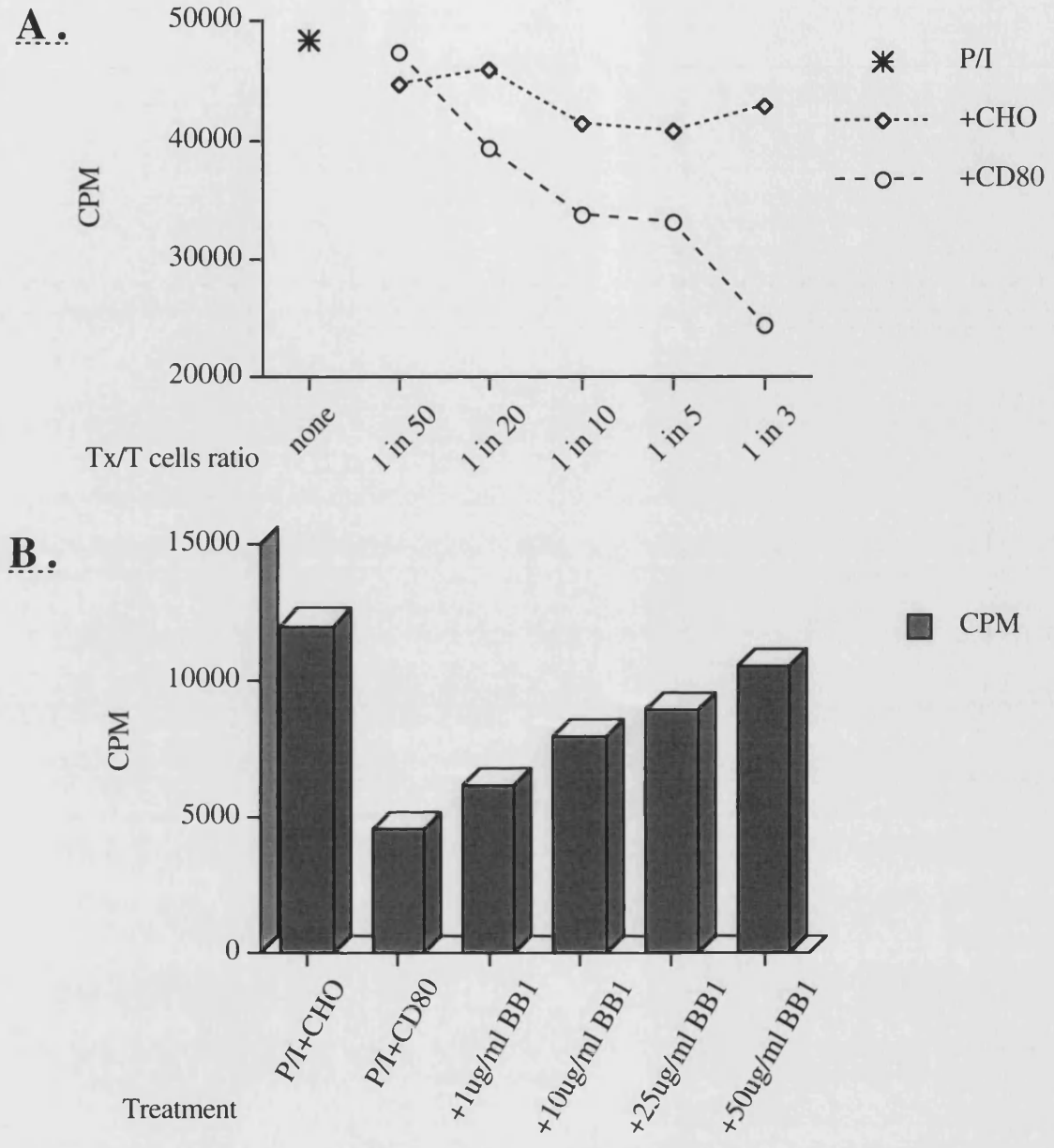

FIGURE 5.2: CD80 negatively regulates PMA and ionomycin T cell responses in a dose dependent manner. Purified human resting T cells were left untreated or stimulated with 5ng/ml PMA and 1μM ionomycin (P/I) alone or with the additional presence of CHO or CHO-CD80 cells. In **panel A**, CHO and CHO-CD80 cells were used at the indicated ratio to T cells. In **panel B**, CHO and CHO-CD80 cells were used at a ratio of 1:3 T cells and the effect of various doses of the anti-CD80 antibody BB1 was examined. Proliferation was measured at 72 hours by the incorporation of ^{3}H-thymidine during an additional 18 hour incubation.

usual. Thus, whilst the proliferation measured at 72 hours may have represented the peak response to P/I, the maximum response to P/I+CD80 may have been earlier and therefore missed. To examine this, kinetic experiments were performed by measuring proliferation at a number of time points. The results from these experiments are shown in **figure 5.3** and indicated that P/I+CD80 proliferative levels were reduced at all time points measured. Both responses peaked at the same time (60 hours) after which they started to decline. Thus the ability of CD80 to downregulate responses appeared due to a distinct mechanism which prevented T cells from proliferating to P/I and not to a more rapid response.

To further examine the possible role of CD28 in this system, cells were stimulated as above and the effect of anti-CD28 antibodies (cross-linked) was compared with that of CD80 (**figure 5.4a**). As expected anti-CD28 antibodies costimulated T cell responses induced by PMA alone, although in this case not as effectively as CD80. When the effect on P/I responses was examined, anti-CD28 antibodies were unable to mimic the CD80 induced downregulation, further supporting the idea that CD28 does not mediate the negative signals induced by CD80. The role of CD28 was finally examined by Fab fragments of the anti-CD28 antibody, which prevent receptor engagement and subsequent signalling. As expected, the ability of CD80 to costimulate PMA responses was blocked by these antibodies, clearly demonstrating that this response was CD28 dependent (**figure 5.4b**). However P/I+CD80 responses were also affected by CD28 Fab and restored to P/I levels. Collectively, these results suggested that whereas CD28 may not be mediating the negative regulatory functions of CD80, it was indirectly important in the process. In respect to that, although according to two recent reports CD28 may not be absolutely required (Fallarino et al., 1998; Lin et al., 1998), others have actually suggested a role for CD28 in the ability of CTLA-4 to negatively regulate activation (Alegre et al., 1996; Finn et al., 1997; Linsley et al., 1992a; Lindsten et al., 1993).

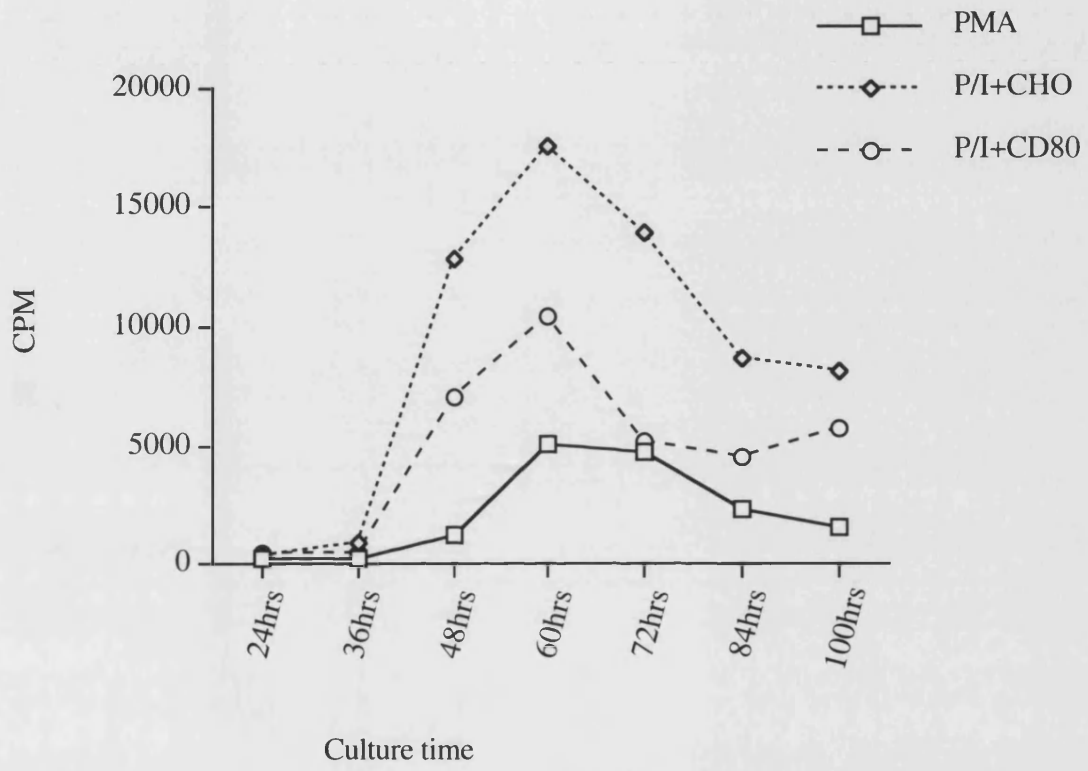

FIGURE 5.3: Time course of P/I+CHO versus P/I+CD80 responses Purified human resting T cells were stimulated with 5ng/ml PMA alone (PMA) or with 1μM ionomycin together with CHO cells (P/I+CHO) or CHO-CD80 cells (P/I+CD80). CHO and CHO-CD80 cells were used at a ratio of 1:3 T cells. Proliferation was measured at the indicated times by the incorporation of ^{3}H-thymidine during an additional 12 hour incubation.

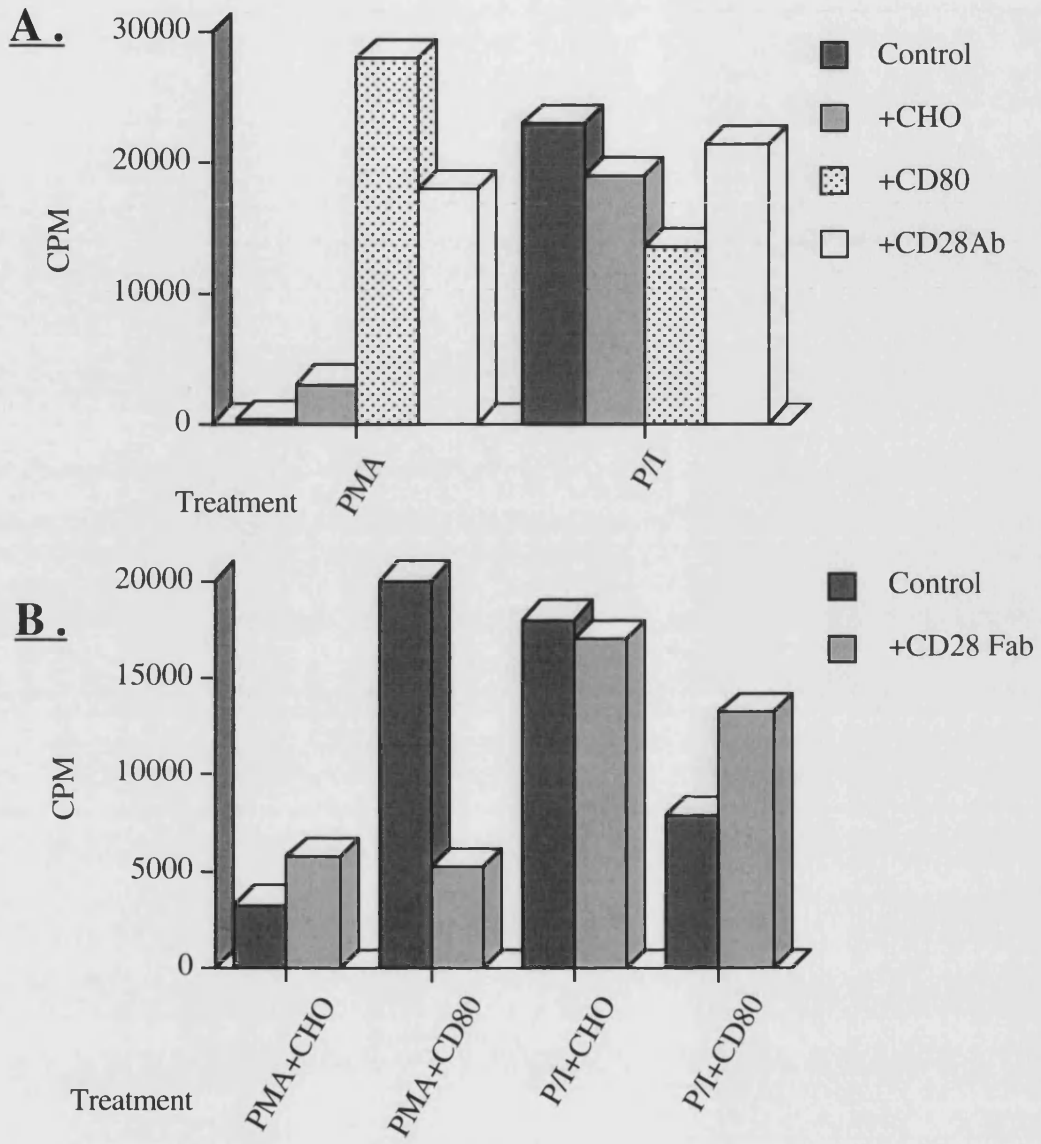

FIGURE 5.4: Role of CD28 in P/I+CD80 responses. Purified human resting T cells were stimulated with 5ng/ml PMA alone (PMA) or together with 1μM ionomycin (P/I) **(Panel A)** and the additional effect of CHO or CHO-CD80 cells (both at a ratio of 1:3 T cells) or 2μg/ml anti-CD28 antibodies (cross-linked with mouse IgG) was examined. In **panel B**, cells were stimulated with 5ng/ml PMA or with 5ng/ml PMA and 1μM ionomycin (both with CHO or CHO-CD80 cells at a ratio of 1:3 T cells) and the effect of 10μg/ml anti-CD28 Fab fragments was examined. Proliferation was measured at 72 hours by the incorporation of ^{3}H-thymidine during an additional 18 hour incubation.

The inability of CD28 antibodies to act in the same manner as CD80, supported the idea that CTLA-4 may be the mediator of P/I+CD80 responses. Similar experiments to the above were therefore performed with anti-CTLA-4 antibodies. Interestingly however, although in some experiments a small effect was observed, cross-linked anti-CTLA-4 antibodies were unable to mimic the effect of CD80 **(figure 5.5a)**. This result was unexpected, but it must be noted that despite the fact that this antibody was able to stain the CTLA-4 receptor on the surface of T cells (see later), a positive functional control was not available. Thus, this result may have been due to the fact that the antibody was not able to signal via the receptor. Alternatively, the inability of anti-CTLA-4 antibody to act similarly to CD80 may result from the simultaneous need of the CD28 receptor, as the results with the CD28 Fab also suggested and as others have also shown (Alegre et al., 1996; Finn et al., 1997; Linsley et al., 1992a; Lindsten et al., 1993). To further examine the role of CTLA-4, blocking experiments with Fab fragments of anti-CTLA-4 antibodies were attempted. As predicted, these antibodies were unable to prevent CD80 from costimulating PMA treated T cells, but resulted in enhanced proliferative responses of P/I+CD80 treated cells **(Figure 5.5b)**. The inability to completely restore the inhibition was probably due to the lack of sufficient antibody available to us. Interestingly, the activation of T cells induced by P/I alone was also enhanced slightly in the presence of CTLA-4 Fab suggesting that endogenous CD80 / CD86 might also be present on activated T cells, and downregulate T cell activation. This is supported by the fact that CD86 on T cells is thought to preferentially bind CTLA-4 and not CD28 (Greenfield et al., 1997), and by reports that suggested that T cells negatively regulate T cell activation when they act as APCs (Pichler and Wyss-Coray, 1994; Chai et al., 1998). Collectively, these results suggest that CD80-CTLA-4 interactions are required for the downregulation of P/I+CD80 responses.

5.2.1.1b: Examination of the CD28 and CTLA-4 surface expression during P/I+CD80 responses

The above evidence suggested the possibility that under conditions of P/I

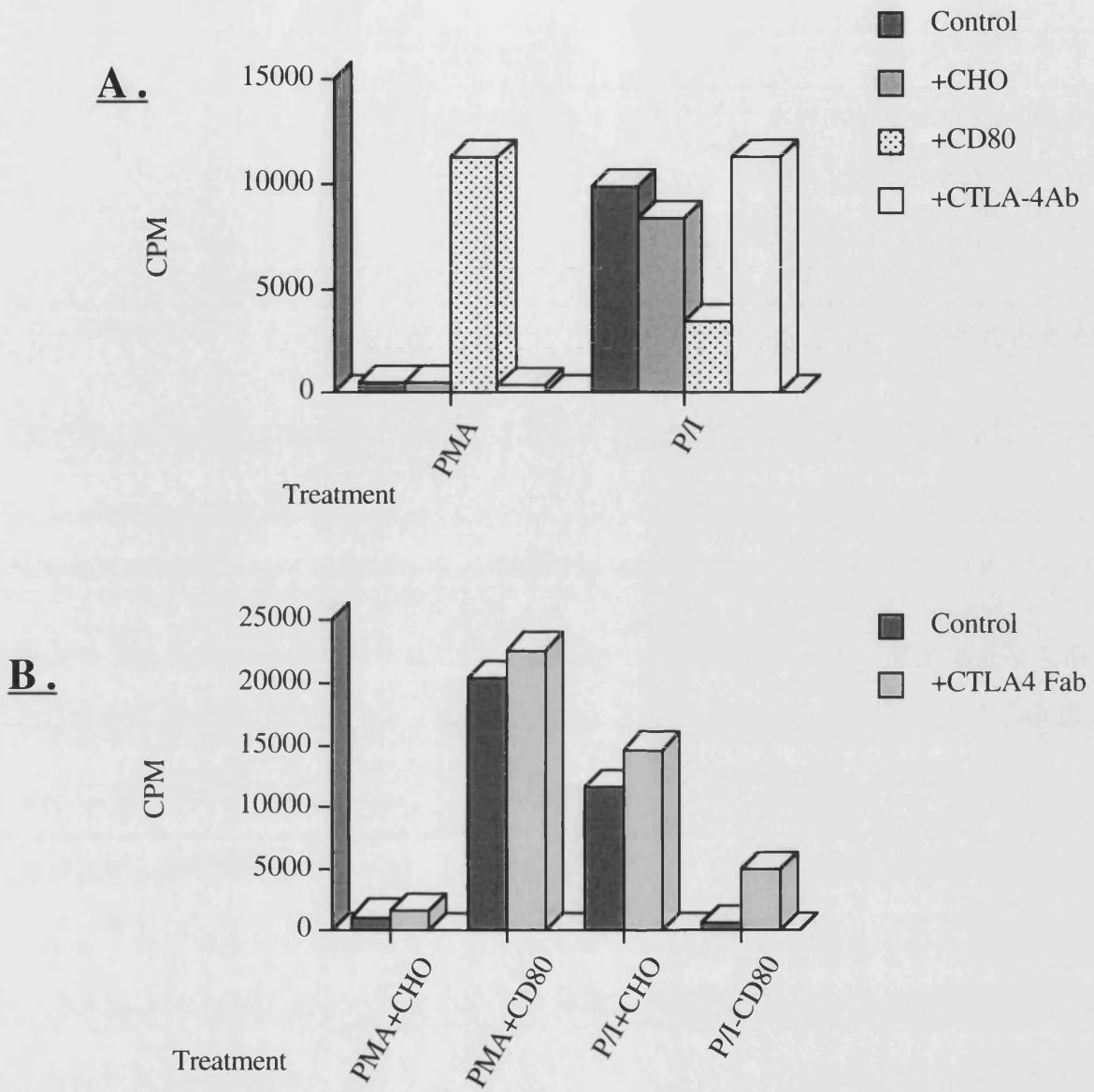

FIGURE 5.5: Role of CTLA-4 in P/I+CD80 responses. Purified human resting T cells were stimulated with 5ng/ml PMA alone (PMA) or together with 1μM ionomycin (P/I) (**Panel A**) and the additional effect of CHO or CHO-CD80 cells (both at a ratio of 1:3 T cells) or 2μg/ml anti-CD28 antibodies (cross-linked with mouse IgG) was examined. In **panel B**, cells were stimulated with 5ng/ml PMA or with 5ng/ml PMA and 1μM ionomycin (both with CHO or CHO-CD80 cells at a ratio of 1:3 T cells) and the effect of 10μg/ml anti-CTLA-4 Fab fragments was examined. Proliferation was measured at 72 hours by the incorporation of ^{3}H-thymidine during an additional 18 hour incubation.

stimulation CD80 may be acting to inhibit responses via CTLA-4. However, they also indicated the importance of CD28 in this process. Since a characteristic of CD28 after engagement with CD80, is its endocytosis and subsequent downregulation (Linsley et al., 1993; Cefai et al., 1998), the levels of this receptor were examined under these conditions. Surface staining with a CD28 antibody clearly showed that the levels of this receptor were high on a resting T cells (**figure 5.6**). Stimulation with PMA alone did not affect these levels and although P/I was able to mediate a slight downregulation of the receptor, the additional presence of CD80 resulted in a dramatic decrease in the levels of CD28. Since CD28 downregulation is an indication of receptor engagement, these results suggested that CD28 is actually being engaged on T cells stimulated with P/I+CD80, and further supported the concept that this receptor plays a role in the ability of CD80 to negatively regulate T cell activation.

The ability of CTLA-4 Fab antibody to partly prevent CD80 mediated downregulation, clearly suggested that this receptor participated in the negative effects of CD80. However, for this to take place P/I stimulation must be able to induce CTLA-4 on the surface of the cells. Surface CTLA-4 is only transient at the first hours of stimulation by repeatedly being exocytosed and then endocytosed back in the cells (Linsley et al., 1996; Zhang and Allison, 1997; Bradshaw et al., 1997; Chuang et al., 1997; Shiratori et al., 1997). Thus, during the first hours of activation CTLA-4 is predominantly intracellular, located in vesicles which are directly targeted to the cell surface (Linsley et al., 1996). For that reason, in the staining studies performed here the usual surface staining protocol was not followed. Instead, T cells were stained with anti-CTLA-4 antibody during the culture period. As a result any antibody that binds CTLA-4 is taken in the cell (with the receptor) via endocytosis. At the end of the culture, intracellular staining detects any endocytosed antibody and therefore any CTLA-4 that would have been present in the surface of the T cells during the culture period. A similar approach by others has detected CTLA-4, as soon as 30 minutes after activation in a calcium dependent manner

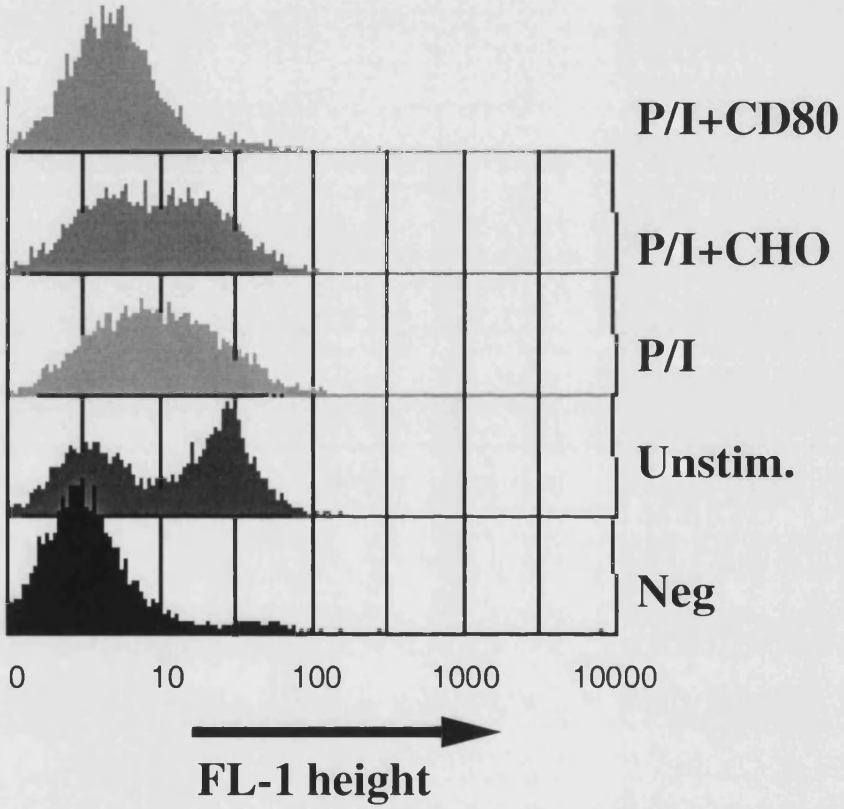

FIGURE 5.6: CD28 expression on activated T cells. Purified human resting T cells were left unstimulated or stimulated with 5ng/ml PMA alone (PMA) or together with 1μM ionomycin (P/I) and the additional effect of CHO or CHO-CD80 cells (both at a ratio of 1:3 T cells). At 48 hours cells were collected and stained for the CD28 receptor on their surface. The negative control represents basal staining levels obtained with the secondary antibody alone.

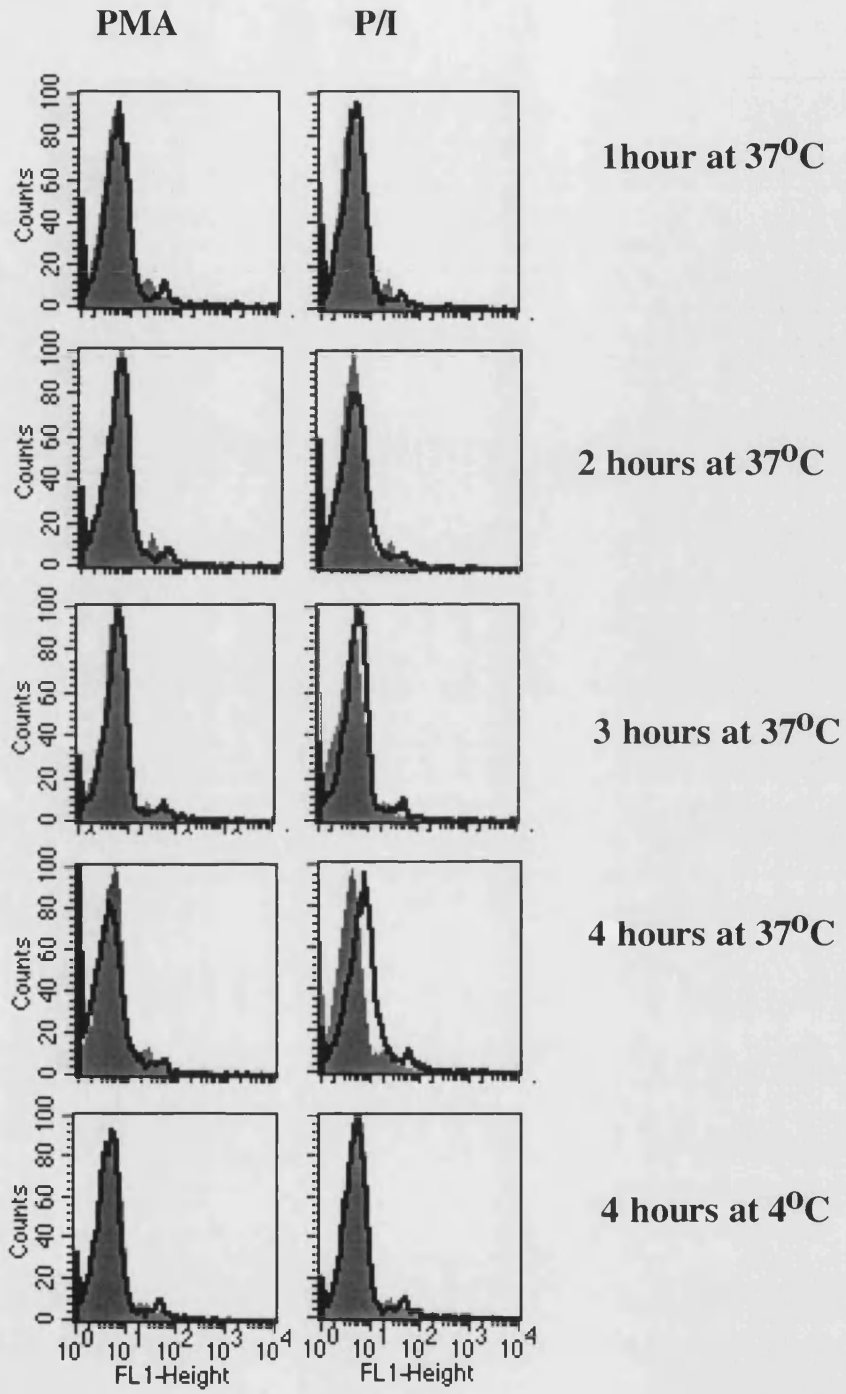

FIGURE 5.7: CTLA-4 surface expression on activated T cells. Purified human resting T cells were stimulated with 5ng/ml PMA alone (PMA) or together with 1μM ionomycin (P/I) and incubated for the indicated times with the CTLA-4 antibody (1μg/ml). At the specified times cells were intracellular stained for any endocytosed antibody (solid line) The negative control (broken line) represents basal staining levels obtained with the control UCHM1 antibody instead of CTLA-4.

(Linsley et al., 1996). In the studies presented here (**figure 5.7**) cells were stimulated for up to 4 hours. However, CTLA-4 was not detected at surface of resting T cells nor T cells activated with PMA alone. Only when cells were stimulated with P/I did CTLA-4 appear in the surface after 4 hours. The same stimulation at 4°C did not result in upregulation of CTLA-4, clearly showing that an active exocytotic cycle is important for the increased CTLA-4 expression. Despite the fact that surface expression of CTLA-4 was limited, this level of expression was in line with other studies indicating low levels of CTLA-4 at the cell surface (Linsley et al., 1992a; Lindsten et al., 1993; Finn et al., 1997; Alegre et al., 1996). Additionally, the ability of CTLA-4 to bind its ligand with high affinity (Linsley et al., 1994; Greenfield et al., 1997) would render these levels functionally significant. Thus these data supported the possibility that CD80 mediated inhibition in the presence of P/I may be due to the expression of utilisation of CTLA-4. In contrast, PMA alone did not promote CTLA-4 surface expression thereby allowing costimulation via CD28 to predominate. Thus, as others have shown (Linsley et al., 1996; Alegre et al., 1996) calcium signals may be vital for the upregulation of the CTLA-4 receptor.

5.2.1.2: An examination the regulation of P/I+CD80 responses and presumably CTLA-4 function by calcium .

The ability of P/I but not PMA alone, to increase CTLA-4 surface expression, suggest that ionomycin and therefore calcium, may be playing a critical role in the CD80 mediated negative regulation. To examine that in more detail the effect of various concentrations of ionomycin, was determined on the ability of CD80 to negatively regulate proliferation of PMA activated T cells. As expected and as shown in the previous chapters, in the absence of ionomycin, CD80 was able to costimulate PMA responses (**figure 5.8**). Even when low concentrations of ionomycin were present this costimulatory ability was not lost. As ionomycin concentration increased however, CD80 costimulation became less potent and at 0.5μM CD80 could not increase P/I signals which are at their peak. Although T cells stimulated with P/I still showed a maximum response at 1μM ionomycin the

presence of CD80 decreased it substantially. At even higher concentrations of ionomycin, although P/I responses became less efficient, the addition of CD80 was able to consistently decrease them further. In fact, as **figure 5.8b** shows, a larger percentage of these responses was downregulated by CD80 as ionomycin concentration increased. Thus, according to these results the presence of a calcium signal is not enough to allow CD80 to downregulate T cells responses, but it has to reach a certain threshold, represented here by 1μM ionomycin. Calcium measurements inside the cells are required to examine this threshold more quantitatively.

Ionomycin elevates calcium in the cell and as a result leads to the activation of a number of calcium dependent proteins including the protein phosphatase calcineurin (Chatila et al., 1998). Since the latter is considered important in T cell activation its role in the responses induced by P/I+CD80 was examined. Cells were stimulated as above with PMA and P/I (with or without CD80) and the effect of CsA was determined. As also shown in chapter 4 the ability of PMA to synergise with CD80 was unaffected by this drug, while P/I stimulation was completely blocked (**figure 5.9**). However, despite its immunosupressive properties, CsA enhanced P/I+CD80 responses and restored the proliferative levels to those observed with PMA+CD80 alone. Thus, according to these results, calcineurin is an important mediator of the ionomycin signals that mediate the ability of CD80 to negatively regulate responses.

5.2.1.3: Examination of the possible mode of CD80 downregulatory activity

5.2.1.3a: CD80 inhibition is most effective early after activation

Previous studies have shown that cross-linked anti-CTLA-4 antibodies can prevent T cell activation by CD3 and CD28, suggesting that CTLA-4 may act early (Walunas et al., 1996a; Krummel and Allison, 1996; Krummel and Allison, 1995). This idea contradicted expression studies which revealed maximal surface CTLA-4 levels approximately 48-72 hours following activation (Alegre et al., 1996; Lindsten et al., 1993; Linsley et al., 1992a). However, more recent findings (Linsley et al.,

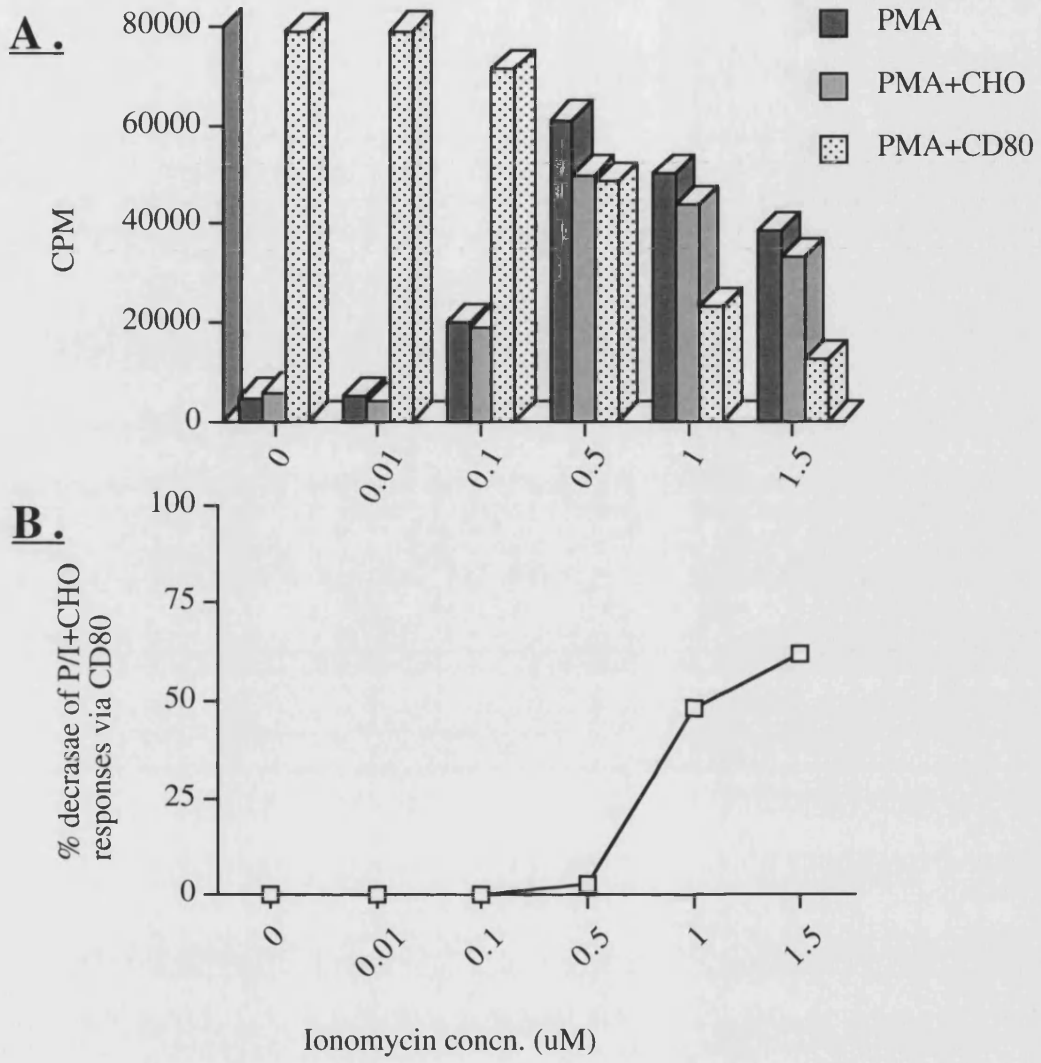

FIGURE 5.8: Role of ionomycin / calcium in P/I+CD80 responses. Purified human resting T cells were stimulated with 5ng/ml PMA alone (PMA) or together with CHO or CHO-CD80 cells (both at a ratio of 1:3 T cells) and the effect of various concentrations of ionomycin was determined. Proliferation **(panel A)** was measured at 72 hours by the incorporation of ^{3}H-thymidine during an additional 18 hour incubation. **Panel B**, represents the percentage downregulation of P/I+CHO responses by the use of CHO-CD80 cells instead.

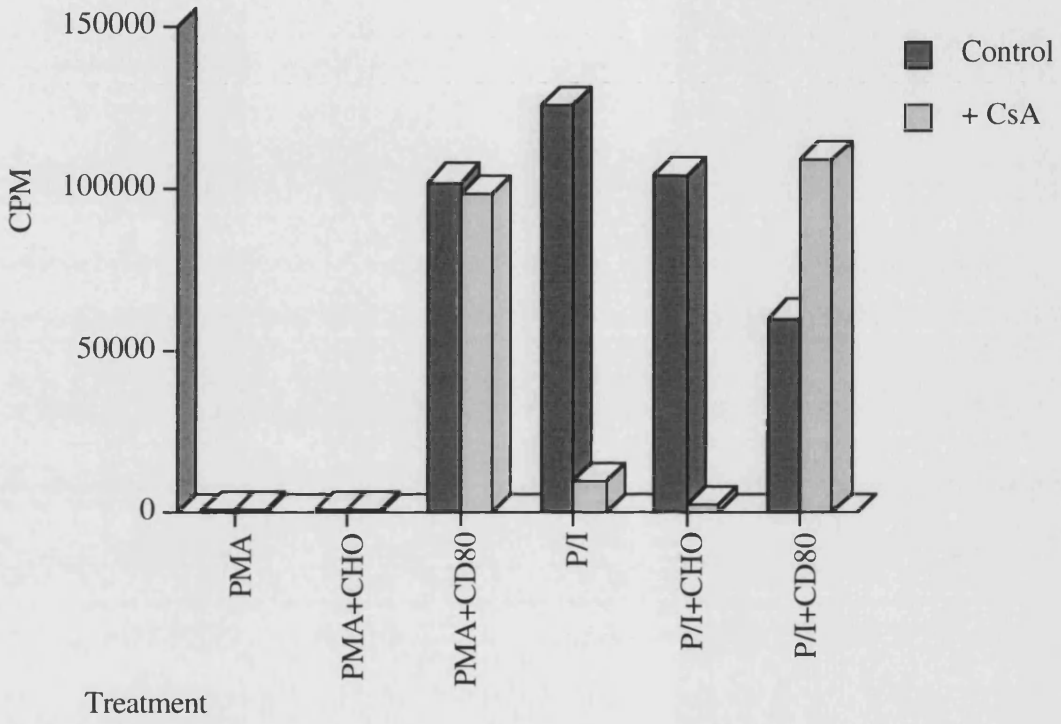

FIGURE 5.9: Effect of CsA on P/I+CD80 responses. Purified human resting T cells were left alone or pre-incubated with 1μg/ml CsA and were then stimulated with 5ng/ml PMA alone (PMA) or with 5ng/ml PMA and 1μM ionomycin (P/I) alone or together with CHO or CHO-CD80 cells (both at a ratio of 1:3 T cells). Proliferation was measured at 72 hours by the incorporation of ^{3}H-thymidine during an additional 18 hour incubation.

1996) and the staining studies above, suggest the presence of CTLA-4 at earlier time points. To further investigate the ability of CD80 to act as a negative regulator of T cell activation, its ability to decrease P/I responses at various time points after activation was examined. For this reason, T cells were activated with P/I and the addition of CD80 transfectants was delayed. As above, adding CD80 at the start of the culture lead to a significant decrease of the proliferative levels (**figure 5.10**). Interestingly, delaying the addition of CD80 by 2 hours resulted in a more profound inhibitory effect. The ability of CD80 to negatively regulate better this way, probably results from the increased CTLA-4 expression during this time. Thus, competition of ligand with CD28 may have swung in favour of CTLA-4. The results in **figure 5.10** however, also indicated that the negative regulatory potential of CD80 becomes less potent with further delays of CHO-CD80 addition. These results suggested that CTLA-4 inhibition can act at an early stage following T cell activation. However, once the cells reach a certain level of stimulation, they can bypass this negatively regulatory mechanism.

5.2.1.3b: CTLA-4 does not promote cell death

The ability of CTLA-4 to act as a negative regulator of T cell activation has been well established, but considerable debate exists about the way it excerts its negative functions. Since some studies have suggested that CTLA-4 may promote apoptosis (Gribben et al., 1995; Scheipers and Reiser, 1998) the viability of T cells activated with P/I+CD80 was compared to the one of P/I+CHO stimulated cells (**figure 5.11a**). In both cases a certain number of cells were dead and stained positive for propidium iodide after 3 days. However, no substantial differences were observed between the two populations, suggesting that the decreased proliferation observed after the addition of CD80 (**figure 5.11b**) is not attributed to death. This is in support with most studies that have not detected increased death after CTLA-4 stimulation and with a recent report that has found CTLA-4 unable to prevent the induction of the anti-apoptotic molecule bcl-X_L (Blair et al., 1998)

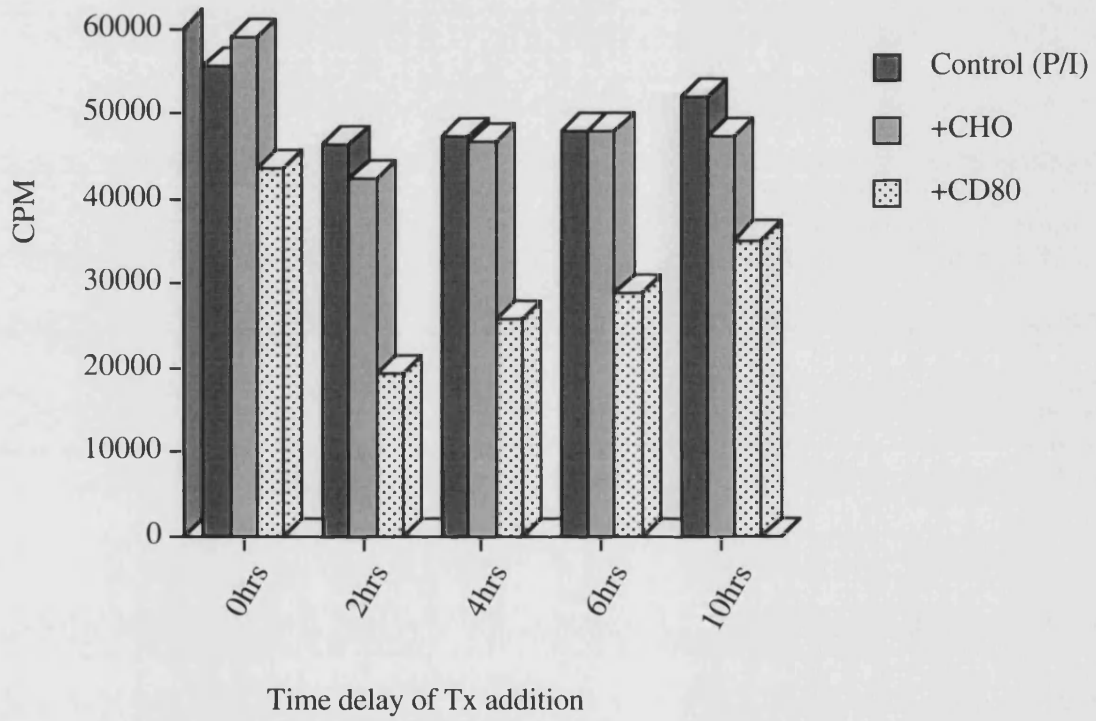

FIGURE 5.10: Effect of delaying CD80 addition on P/I+CD80 responses. Purified human resting T cells were stimulated with 5ng/ml PMA and 1μM ionomycin (P/I) together with CHO or CHO-CD80 cells (both at a ratio of 1:3 T cells) added at various times after the initial P/I stimulation. Proliferation was measured at 72 hours after the initial P/I stimulation by the incorporation of ^{3}H-thymidine during an additional 18 hour incubation.

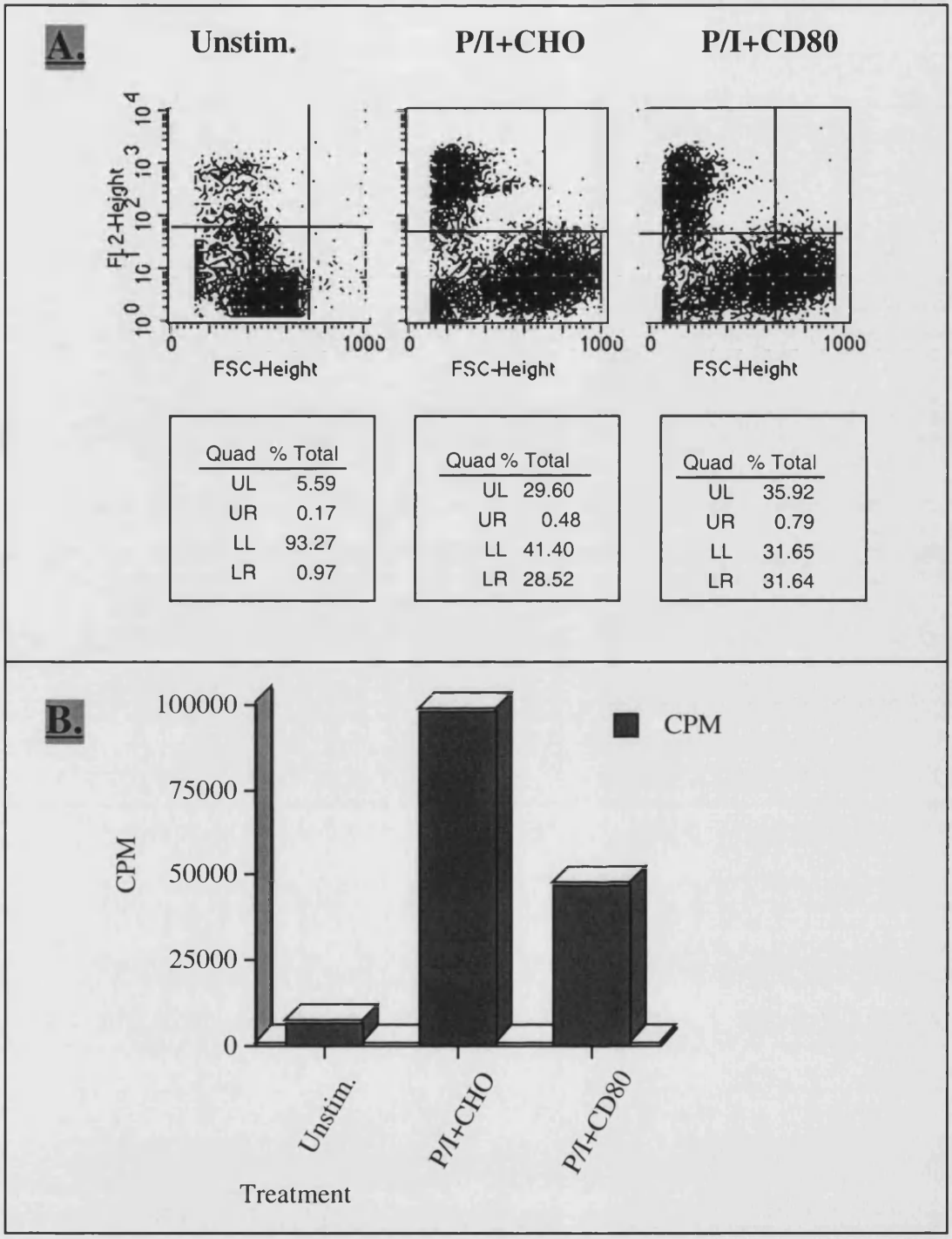

FIGURE 5.11: Viability of P/I+CD80 activated T cells. Purified human resting T cells were stimulated with 5ng/ml PMA alone or with 5ng/ml PMA and 1μM ionomycin (P/I) together with CHO or CHO-CD80 cells (both at a ratio of 1:3 T cells). Cell viability was determined by propidium iodide staining (FL-2) **(panel A)**. **Panel B** shows the proliferative responses of the corresponding cells measured at 72 hours by the incorporation of ^{3}H-thymidine during an additional 18 hour incubation.

5.2.2: Other experimental systems that allow the examination of the negative regulatory potential of CD80.

5.2.2.1: Inhibition of endogenous CD80 / CD86 enhances responses of PBMCs to P/I in a CD28 and CTLA-4 dependent manner.

In the experimental system presented above and throughout this thesis, CHO-CD80 cells were used as artificial APCs on resting T cells. In an attempt to see if "true" APCs with endogenous CD80 and CD86 could downregulate T cell responses as well, whole PBMC populations, were used. Based on the findings above, CD80 / CD86 expressed by APCs (i.e. monocytes, B cells etc.) within this population, would be capable of downregulating the proliferative responses to P/I. Consequently blocking CD80 and CD86 with CTLA-4-Ig would potentially enhance these responses. Indeed as **figure 5.12a** shows, PBMCs responded weakly to P/I but proliferated better in the additional presence of CTLA-4-Ig but not in the presence of control mouse Ig. This result was in stark contrast to the immunosupressive effects of CTLA-4-Ig seen during CD28-dependent costimulation but strongly supported the hypothesis that under conditions of P/I stimulation a substantial response to natural ligands results from negative regulatory signals. Blocking of CD80 and CD86 reveals these negative regulatory functions. To verify that these negative signals are mediated by CTLA-4 additional studies were performed via blocking experiments. Similarly to the results obtained with resting T cells, PBMC responses to P/I were enhanced in the presence of anti-CTLA-4 Fab but also anti-CD28 Fab antibodies (**figure 5.12b**). Thus, both receptors seem to be involved in the final determination of the PBMC responses to P/I. Interestingly the same studies revealed that endogenous CD80 / CD86 is unable to costimulate PMA responses. The most likely reason for this result is that CD80 / CD86 are present at low levels in freshly purified PBMC populations, a parameter which has been suggested to promote CTLA-4 activity on T cells but not CD28.

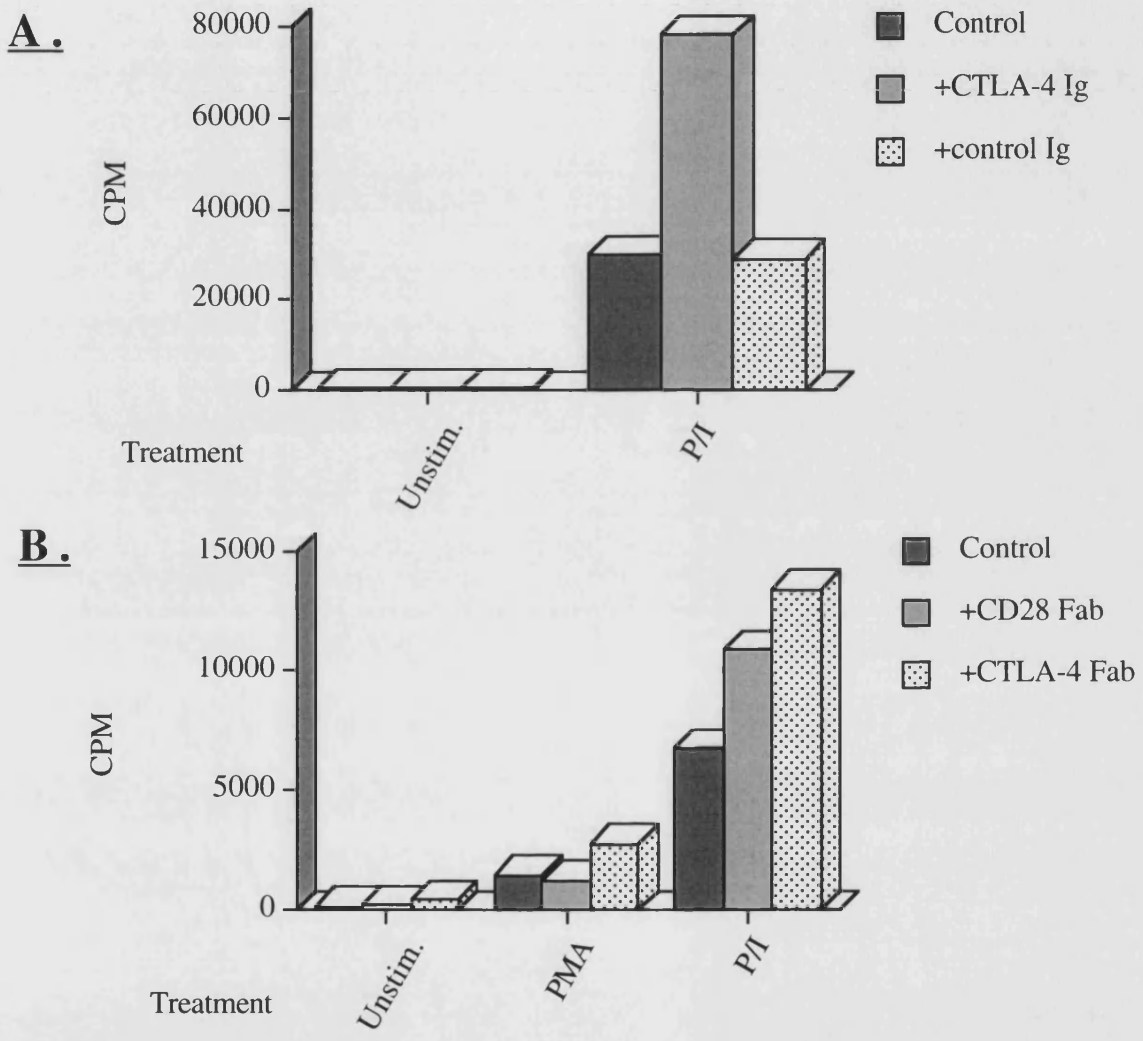

FIGURE 5.12: Effect of endogenous CD80 / CD86 and CD28 and CTLA-4 on P/I responses of PBMCs. Purified human PBMCs were left alone or stimulated with 5ng/ml PMA or 5ng/ml PMA and 1μM ionomycin (P/I). The effect of 1μg/ml CTLA-4Ig **(panel A)**, 10μg/ml Fab fragments of anti-CD28 **(panel B)** 80μg/ml Fab fragments of anti-CTLA-4 **(panel B)** was examined. Proliferation was measured at 48 hours by the incorporation of ^{3}H-thymidine during an additional 18 hour incubation.

5.2.2.2: Strong signals via CD3 and CD28 result in responses that are negatively regulated by CD80

The results presented so far suggested that the strength of the calcium signal plays an important role in the regulation of positive or negative effects that CD80 mediates. Since calcium is a mediator of TCR / CD3 signalling, this may mean that the strength of the signals initiated by these receptors may play a crucial role in the final outcome of T cell activation. Specifically, according to the results obtained with ionomycin, high strength TCR / CD3 signals will be expected to promote more CTLA-4 activity than low strength signals. In relation to that, data presented in chapter 3 suggested that CD80 costimulation was less effective at high concentrations of CD3 antibody. It is therefore possible that this may result from more CTLA-4 engagement and therefore more negative signalling by CD80, that competes with and antagonises costimulatory signals after CD28 engagement by the same ligand. To examine this possibility T cells were activated with various concentrations of anti-CD3 antibody and the effect of CD80 was compared with the one induced by the anti-CD28 antibody. Since the antibodies bind only CD28, they would be expected to induce only positive signals and therefore avoid this competition with negative signals via CTLA-4. Supporting this, the data in **figure 5.13** showed that T cell activation with CD3 and CD28 antibodies peak at the optimal concentrations of CD3 (1μg/ml) and plateau after that. In contrast CD80 responses followed a bell shaped curve, peaking at similar CD3 concentrations but starting to fall after that. Thus, as predicted these results suggest that when T cells are activated by strong CD3 stimuli, CD80 performs some negative regulatory functions and as a result decreases its costimulatory potential. More experiments that utilise anti-CTLA-4 Fab antibodies are required to further examine these data. If the interpretation of these results is correct, the addition of these blocking antibodies would be expected to restore the strength of the proliferative levels to the ones observed by CD28 antibodies.

Apart from implicating calcium as an important regulator of T cell activation, the

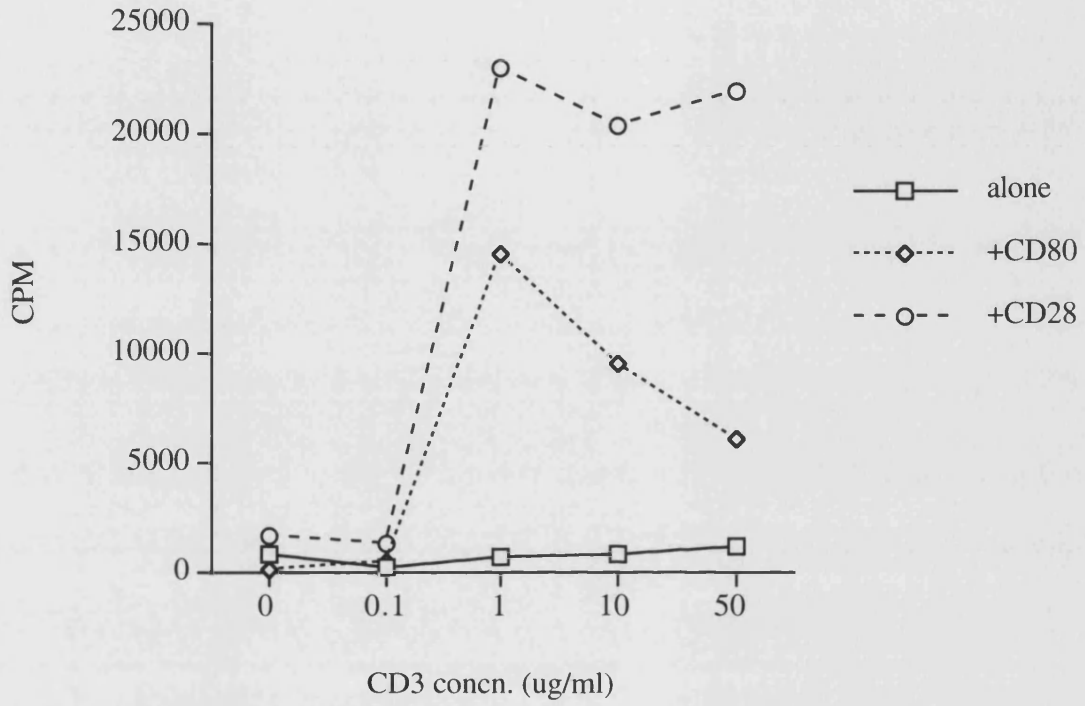

FIGURE 5.13: Effect of varying CD3 concentrations on the costimulatory ability of CD80 and CD28 antibodies. Purified human resting T cells were left unstimulated or stimulated with varying concentrations of CD3 antibody (plate coated for 18 hours) and additionally treated with either CHO-CD80 cells (+CD80) or 2μg/ml soluble CD28 antibodies (cross-linked with mouse IgG) (+CD28). Proliferation was measured at 72 hours by the incorporation of ^{3}H-thymidine during an additional 18 hour incubation.

data obtained with the responses of P/I+CD80 treated T cells, also suggested that CD28 may be participating in the promotion of CD80 mediated negative regulation. To examine this further the effect of increasing CD28 engagement on CD80 function was determined. Thus, T cells were stimulated with high concentration of anti-CD3 antibody in order to promote CTLA-4 function as suggested above and with various concentrations of anti-CD28 antibody. The additional effect of CHO-CD80 cells on these stimulations was then examined. As **figure 5.14** showed CD3 activated T cells were costimulated with CD80 in the absence of anti-CD28 antibodies. The presence of low levels of CD28 engagement (0.1μg/ml CD28Ab), still allowed CD80 to costimulate T cells further, but optimal CD28 activation (1μg/ml CD28Ab) was able to activate T cells without any additional positive effect by CD80. Finally, whereas stronger engagement of CD28 (10 or 100μg/ml CD28Ab) costimulated CD3 activated T cells, the additional presence of CD80 actually negatively regulated the responses. Clearly, although more data are required in order to establish the CD80 / CTLA-4 specificity of this signal, these results suggested again, that CD28 plays a role in the regulation of the negative signalling capability of CD80. Supporting this, similar studies with antibodies have shown that in order for anti-CTLA-4 antibodies to negatively regulate activation more effectively, T cells have to be treated with high doses of anti-CD3 and anti-CD28 antibodies. (Krummel and Allison, 1996; Walunas et al., 1996a; Krummel and Allison, 1995). Thus, the general state of the activation of the cells may control the negative regulatory signals that CTLA-4 mediates.

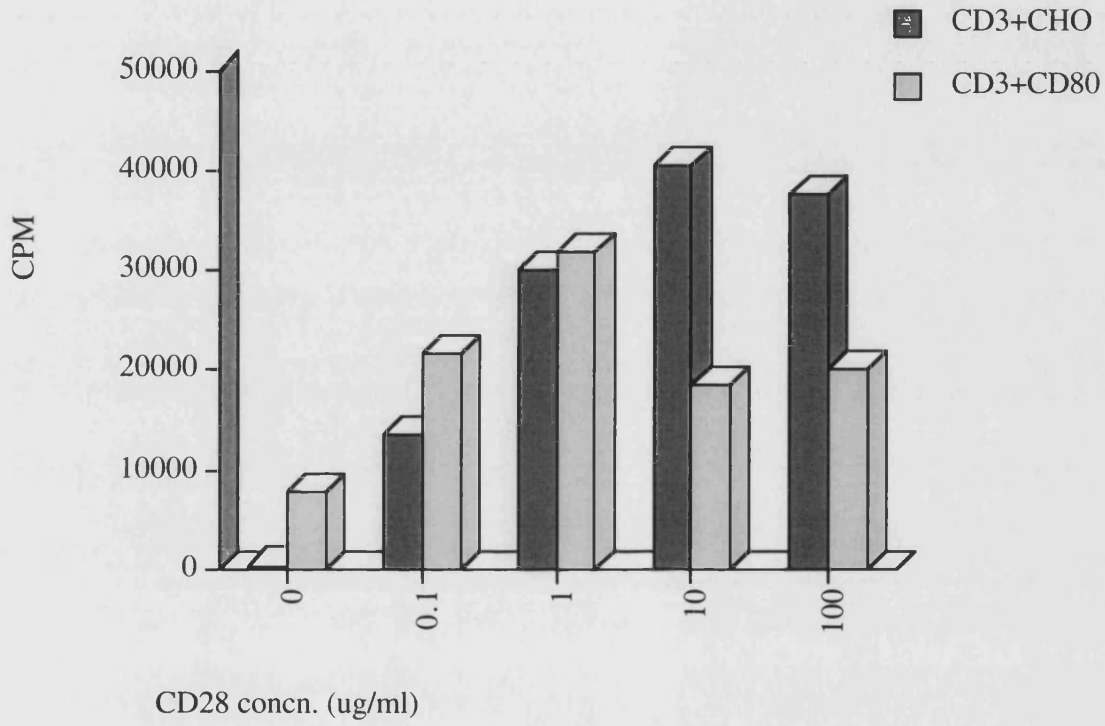

FIGURE 5.14: Effect of varying CD28 stimulation on the negative regulatory ability of CD80. Purified human resting T cells were left unstimulated or stimulated with 10μg/ml CD3 antibody (plate coated for 18 hours) and varying concentrations of soluble CD28 antibodies (cross-linked with mouse IgG). The additional effect of CHO-CD80 cells was compared to that of CHO cells. CHO cells and CHO-CD80 cells were utilised at a ratio of 1:3 T cells. Proliferation was measured at 72 hours by the incorporation of ^{3}H-thymidine during an additional 18 hour incubation.

5.3: DISCUSSION

The role of CTLA-4 as a negative regulator of T cell activation has been convincingly documented both *in vivo* and *in vitro* (Walunas et al., 1994; Krummel and Allison, 1995; Waterhouse et al., 1995). However whilst it is clear that antibodies directed to CTLA-4 can efficiently inhibit T cell activation, similar studies using natural ligands have not been forthcoming despite this being a central prediction. On the contrary, where T cell activation has been studied using CD28 / CTLA-4 ligands, only costimulatory functions are observed (Linsley et al., 1991a; Sansom et al., 1993; Edmead et al., 1996). Clearly however the observed outcome in these experiments is the combined result of positive and negative influences on T cell activation by these ligands. Distinguishing between the two types of signals and therefore separating CD28 and CTLA-4 functions has been difficult and has hampered natural ligand studies when CTLA-4 is concerned. However as the results show here, CD80 can costimulate PMA activated human T cells but is able to negatively regulate the costimulation independent activation of human resting T cells with P/I. Additionally, whereas the costimulatory activity of CD80 utilises CD28, negative regulation of P/I responses requires CTLA-4. Importantly, this is the first demonstration of both positive and negative effects of the natural ligand CD80 on T cell proliferation.

Interestingly, the results here reveal that whilst CTLA-4 is clearly involved in suppressing P/I responses, CD28 interactions are also important in promoting downregulation. Given that CD80 binds to both ligands this is perhaps not surprising although it might appear paradoxical that CD28 appears to be involved positively in PMA + CD80 responses yet negatively in P/I-CD80 responses. However, the fact that CD28 antibodies can not mimic the effect of CD80 suggests that the role of CD28 is not actively negative in these experiments. Instead, CD28 interactions may be important in promoting CTLA-4 expression and function. There are several possible mechanisms for this. Firstly, it is highly likely that CD28 is

involved in enhancing the contact of CD80 transfectants with the T cells and therefore permit CTLA-4 interactions. A second possibility is that CD28 signals may actually be a requirement for effective expression of CTLA-4 as has been previously suggested (Alegre et al., 1996; Finn et al., 1997; Linsley et al., 1992a; Lindsten et al., 1993). Thirdly, CD28 downregulation as a result of ligand engagement may also be involved in shifting the balance towards CTLA-4. Thus, although CD80 interacts with CTLA-4 and not CD28 in order to actively mediate its negative signals, CD28 seems to be involved indirectly by aiding the ability of CTLA-4 to interact with CD80. This possibly reflects an initial requirement for CD28 engagement followed by CTLA-4 inhibition.

Several lines of evidence presented in this chapter, indicate calcium as an important factor that regulates the costimulatory or inhibitory potential of CD80. Notably, only P/I but not PMA stimulated T cells are negatively regulated by CD80. Interestingly a certain strength of calcium signal is required for CD80 to downregulate P/I responses. Low ionomycin concentrations can synergise with PMA and even be costimulated further by CD80. It is not until a threshold of ionomycin concentration (between 0.5-1μM) is utilised that CD80 starts to downregulate responses. These results are also supported by similar titrations using CD3 antibodies, since these elevate calcium in the cells after ligating CD3 (Nakano et al., 1993; Sarkadi et al., 1991). Again an optimal CD3 concentration seems to be reached until which CD80 is able to increasingly costimulate responses. However as this concentration of antibody is increased further CD80 becomes a less potent costimulator. In contrast CD28 antibodies do not loose their costimulatory ability. Assuming that the engagement of CTLA-4 in the only difference between these two CD28 agonists, the ability of CD80 to act negatively via CTLA-4 is the most likely explanation of these results. Further studies using CTLA-4 blocking antibodies are required in order to verify this.

The ability of calcium to dictate the negative regulatory effect of CD80 via CTLA-4 is most likely explained by the fact that CTLA-4 expression is dependent on a sustained calcium signal. Clearly, P/I (but not PMA) stimulation leads to detectable CTLA-4 at the cell surface. This is in line with data from other studies (Linsley et al., 1996; Alegre et al., 1996; Lindsten et al., 1993) which have demonstrated a requirement for calcium signalling in both messenger RNA induction and in surface expression. In particular, Linsley *et. al.* (Linsley et al., 1996) showed that ionomycin was the most potent signal at inducing surface expression of CTLA-4, whilst others have shown increased CTLA-4 messenger RNA following P/I stimulation of resting T cells (Lindsten et al., 1993; Freeman et al., 1992). Thus, calcium levels in the cell after activation may turn out as a key regulator of CTLA-4 function. Importantly, the addition of CsA leads to a reversal of CD80 induced down regulation and restores proliferation to the level of PMA and CD80 combined. This finding is highly consistent with the effects of CsA on CTLA-4 expression which has been described in two previous studies (Finn et al., 1997; Alegre et al., 1996). It is unclear how calcineurin (the target of CsA) is exactly involved in the process, but there are three possible ways. Firstly, activation of NFAT by calcineurin may enhance the transcriptional activity of the CTLA-4 promoter, which as others have shown contain a corresponding DNA binding site (Finn et al., 1997). This would increase CTLA-4 gene expression and subsequently the levels of this receptor in the cells. Secondly, calcineurin may be implicated in the exocytotic pathway and therefore the rise of CTLA-4 in the surface. In this respect, calcium is an important regulator of vesicular transport mechanisms, including exocytosis (Rothman, 1994; Darnell et al., 1990; Linsley et al., 1996) and more specifically, studies with gastric chief cells, have shown calcineurin to play a vital role in the secretion of proteins via exocytosis (Raufman et al., 1997). Unfortunately the key experiment, examining the effect of CsA in CTLA-4 expression after P/I stimulation was not performed here. Finally, although supporting evidence does not exist, calcineurin may be actively involved in the negative signals that CTLA-4 mediates.

It must be noted however that calcium is probably not the only factor that determines whether CD80 will costimulate or downregulate T cell activation. Other signals are probably also important. In respect to that P/I signals are downregulated only when PMA is also utilised at high concentrations. As shown in chapter 3, low concentrations of PMA are costimulated by CD80 despite the additional presence of 1μM ionomycin. Thus, a general state of activation may determine the role of CD80. In further support to this, the data presented here also suggest that strong CD28 signalling can also promote negative regulation by CD80. Collectively, all these results suggest that CTLA-4 may act to downregulate and limit responses that would have resulted to overactivation and possibly cell death after a strong stimulus.

Other non signalling factors may also affect domination of positive or negative effects by CD80. The levels of CD28 and CTLA-4 expression may for example play a crucial role. P/I stimulation for example increase CTLA-4 but at the same time slightly decreases CD28 expression levels. The additional presence of CD80 dramatically drops the level of CD28 within hours of stimulation via receptor endocytosis (Linsley et al., 1993; Cefai et al., 1998). In contrast engagement of CTLA-4 by CD80 is suggested to induce phosphorylation at YVKM motif of the receptor which inhibits interaction with the AP50 protein of clathrin coated pits that aid endocytosis (Zhang and Allison, 1997; Bradshaw et al., 1997; Chuang et al., 1997; Shiratori et al., 1997). Thus the combination of P/I+CD80 may serve to increase CTLA-4 expression whilst simultaneously decreasing CD28 expression, strongly favouring CD80/CTLA-4 interactions. Similar regulation may also exist for the CD28/CTLA-4 counter-receptors CD80 and CD86. It has been suggested (Chambers et al., 1996) that low levels of CD80 may enhance CTLA-4 activity and this is attributed to the ability of the latter to engage CD80 / CD86 with higher affinity and therefore dominate these interactions despite its lower levels on the T cells. The ability of the low levels of CD80 / CD86 on freshly isolated PBMCs, to potently negatively regulate T cells activated with P/I supports this. Interestingly this concept seems to contradict the thought that CD28 promotes CTLA-4 function.

However it is possible that this mechanism ensures that low strength CD28 signals are also prevented from costimulating T cells unproductively. In other words, the role of CTLA-4 in an immune response would be to prevent cells from activating non-optimally. Thus inhibition of low antigenic stimuli may avoid the unproductive mounting of an immune response. In contrast inhibition of high antigenic stimuli may be performed by CTLA-4 in order to prevent subsequent overactivation that may even lead to cell death.

Another question that concerns the action of CTLA-4 is its time of action. Our results also demonstrate that CD80 inhibition is most effective approximately 2 hours after PI activation. This time delay may be explained by the requirement for *de-novo* CTLA-4 synthesis and transport to the cell surface since CTLA-4 mRNA is not observed in resting T cells (Freeman et al., 1992) but is rapidly induced following PI stimulation. However our results also suggest that after this period CTLA-4 inhibition becomes less effective with further delays of CD80 addition following PI stimulation, possibly indicating that once cells have been committed to cell cycle CTLA-4 ligation is less effective at inhibiting responses. Thus it appears that CTLA-4 may act as an "off switch" rather than a "brake" for T cell proliferation. In support of this interpretation we have observed only stimulatory effects when challenging previously activated T cell blasts with CD80 transfectants (Edmead et al., 1996) indicating that once T cells are proliferating CD28 costimulation may predominate.

Interestingly, during these studies we observed that the level of inhibition of responses by CD80 was somewhat variable (between approximately 30% and 90%). One explanation for this variability is the fact that proliferation analysis measures the net result of a balance between costimulatory and inhibitory outcomes mediated by CD28 and CTLA-4. Thus, an overall inhibitory result is only revealed when inhibition outweighs costimulation. In contrast to CTLA-4 antibody-based studies natural ligands are agonists of both costimulation and inhibition. Our experience

suggests that under conditions of limited stimulation, addition of CD80 is generally costimulatory, whereas under increased stimulation a negative effect is revealed. Thus, more potent signals appear to promote greater CTLA-4 expression and thus influence the balance in favour of CTLA-4 ligation as has also been suggested by others (Linsley et al., 1996; Alegre et al., 1996). This concept, that high intensity signals are more susceptible to modulation by CTLA-4 is somewhat counterintuitive since CTLA-4 might therefore potentially inhibit higher affinity T cell interactions. However, similar mechanisms for screening out high affinity interaction appear to be used in thymic selection (Ashtonrickardt et al., 1994) to remove potentially autoreactive clones, and such a use of CTLA-4 peripherally may represent a similar protective mechanism from the consequences of autoimmunity as indicated by CTLA-4 deficient mice (Waterhouse et al., 1996). Overall our studies reveal both positive and negative influences of CD80 in the regulation of T cell proliferation, and provide new insights as to how the decision between the use of CD28 and CTLA-4 is made.

CHAPTER 6

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

The initial suggestion of a two signal model of T cell activation initiated intense investigation that lead to the identification of various receptors that could play the role of the second signal during T cell activation. However, CD28 has been widely accepted as the most important receptor that synergises with the presentation of the antigen. Two factors contribute to this. Firstly, CD28 ligation and signalling has been found able to rescue T cells from the anergic state (or paralysis as it was initially known). Secondly, CD28 has been found to be able to act *in trans* with the antigenic signal, clearly showing that its function is not simply restricted on increasing the adhesion between the T cell and the antigen presenting cell. Despite this, our knowledge and understanding of T cell activation is far from complete. In addition to CD28, a homologous receptor, CTLA-4 (CD152), has been identified. It has become apparent the last few years that this receptor acts as a negative regulator of T cell activation, despite the fact that it interacts with the same ligands as CD28. Thus, a mixture of positive and negative signals is now believed to determine the final outcome of an antigenic simulation.

The work presented in this thesis has examined the costimulatory properties of CD80 and came to the surprising conclusion that its ligation to CD28 initiates essential signals that support proliferation of T cells, but under some circumstances without the need of IL-2. This finding was surprising not only because of the general proliferative potential of IL-2, but also because of the well documented role of IL-2 in the avoidance of T cell anergy. Clearly, the stimulation protocol utilised in the studies presented here was not physiologic, since PMA was used to initiate TCR signalling cascades. However, it is important to note that the IL-2 independent proliferation that we observed in the human naive T cells is not seen when PMA acts alone but instead requires the additional ligation of CD28. In addition, it is important to understand the physiologic relevance of such IL-2 independent T cell activation. In respect to this, a number of studies support the concept that IL-2 is not

as vital for T cell activation as was initially thought. In particular, IL-2 deficient mice have been characterised by an autoimmune disorder and the presence of $CD4^{+ve}$ T cells displaying the activated / memory phenotype (i.e. $CD69^{high}/CD62^{low}$). Significantly, this phenotype is not seen in thymocytes suggesting that the cells reach such a state after an antigenic challenge in the periphery. Furthermore recent studies have shown that these $CD4^{+ve}$ cells are able to proliferate in response to CD28 costimulation. More specifically, LPS has been suggested to promote the ability of CD28 to mediate T cell activation in the absence of IL-2. Thus, it is clear from these studies that T cells can respond to CD28 costimulation, survive and proliferate without IL-2. Clearly, these findings suggest a rethink on the role of IL-2 as a mediator of the CD28 costimulatory functions and lead to the question concerning the identity of the cytokines that may support these functions.

Given the above data it will be important to identify other T cell cytokines that may mediate these functions in the future. The results presented in chapter 4 suggested that the supernatants of human T cells stimulated with PMA+CD80 are capable to induce proliferation, suggesting the presence of a soluble factor. An attempt to identify such a factor with RT-PCR studies have not resulted in any known cytokine as a potential candidate however. IL-13, the cytokine that was induced by PMA+CD80 in a CsA resistant fashion is also not a suitable candidate due to the fact that it is not characterised as a T cell proliferative factor. Nevertheless, this result clearly shows that there are cytokines that can be regulated under these conditions. However it must be noted that the ability of PCR to detect very low amount of RNA that may not be high enough to significantly affect proliferation of the T cells, poses the possible risk of artefacts. A less sensitive approach for the identification of cytokines at the RNA level would be to perform northern blot analysis which detect more significant amounts of RNA. However, even these assays may not be the best approach due to the differences observed between mRNA levels and secretion of a cytokine. In this respect ELISA assays will be a more direct

approach for the identification of such a proliferative factor. However, these assays will be unable to find the factor responsible if the actual target of CD28 signalling is a novel proliferative molecule. A cloning approach, to identify such a factor is now underway in our laboratory, to address the feasibility of expression cloning in COS cells.

As with the CD28 receptor most studies examining the function of CTLA-4 have used antibodies for the receptor. These reports have clearly established the negative regulatory potential of CTLA-4 and the *in vivo* studies have further verified them. However an important aspect of the CD28 / CTLA-4 costimulatory system is that although the two receptors have opposite effects they utilise the same ligand. It is therefore obvious that one of the most important aspects of the regulation will be the competition for ligand binding. The use of antibodies bypasses this form of regulation and may therefore yield unphysiologic results. The data presented here are the first functional study showing the ability of the natural ligand CD80 to not only act as a costimulator of T cell responses but also negatively regulate T cell activation. In the model presented in chapter 5, it is shown that CD80 can negatively regulate T cell responses that are induced by the costimulation independent activation by PMA and ionomycin.

One important finding of the studies presented was the ability of CD80 to negatively regulate T cell activation only in the presence of a calcium / calcineurin dependent signal. Importantly, only high concentrations of ionomycin or anti-CD3 antibodies were able to promote the ability of CD80 to act as a negative regulator of T cell activation. Since calcium is a parameter that characterises signalling from the TCR, this finding suggests that the strength of the antigenic challenge may play a crucial role in determining the final outcome of the T cell responses. It is therefore possible that CTLA-4 acts to limit the effect of strong antigenic stimuli that would otherwise lead to overactivation of the T cells and subsequent unwanted immune responses.

The ability of CD28 and CTLA-4 to ligate the same ligands but still act opposite in the regulation of T cell activation suggests that a competition must be present for the binding of the ligands by the two receptors. Thus, the expression of CD80 / CD86 on the APCs and the levels of CD28 / CTLA-4 on the T cells may play an important role in the final outcome of T cell activation. Thus, an abundant but low affinity receptor (CD28) competes with a scarce but high affinity receptor (CTLA-4) in order to positively or negatively control T cell activation. Due to the high affinity of CTLA-4 for both the ligands, it has been suggested that low levels of CD80 and / or CD86 may preferentially interact with CTLA-4. In respect to this, it is interesting that the levels of CD80 / CD86 are low prior to activation, suggesting that CTLA-4 may act to keep the T cells in a resting state and therefore aid tolerance. It is therefore an attractive possibility that apart from limiting T cell activation after strong stimuli, CTLA-4 may also prevent the initiation of unnecessary responses that may otherwise occur during brief or weak antigenic encounters that are in fact harmless.

Clearly many discrepancies exist on the mode of action of CTLA-4 with some data supporting a role for anergy, others that it promotes apoptosis and others that it simply acts as a "stop" signal in cell cycle progression. All these discrepancies suggest that the role of CTLA-4 and its mode of function may not be the same in all cases and that various parameters that also participate in T cell activation may additionally determine CTLA-4 function. In this respect, IL-2 has also been implicated in promoting CTLA-4 function. It is therefore interesting that as mentioned above, T cells stimulated with PMA+CD80 which are characterised by strong proliferation are suggested not to express CTLA-4. It is therefore possible that the absence of CTLA-4 in these cells may partially aid proliferative responses without IL-2.

Clearly, more work is required in order to fully understand the role and function of CTLA-4 and a number of outstanding questions concerning CTLA-4 still remain. Is

CTLA-4 a universal downregulator of the T cell immune system or does it only affect specific T cells? If the latter is true, it will be important to determine the make up of these types of cells. Another important question is whether CTLA-4 is an inhibitor of TCR or CD28 signals. Clearly, costimulation can be prevented in both cases. However since other costimulatory molecules apart from CD28 do exist it is important to investigate whether CTLA-4 antagonises CD28 costimulation specifically or whether it is able to antagonise the effects of other costimulatory molecules as well. Finally the ability of both CD28 and CTLA-4 to interact with two different ligands prompts an investigation in to possible differential ligand effects on CTLA-4 function. Small differences in the binding affinities of CD80 and CD86 with CTLA-4 do exist and substantial differences in terms of expression levels and localisation in the cells suggest that important functional differences in the action of these two molecules may also exist.

Deciphering the mode of action and function of CD28, CTLA-4 and other possible regulators of antigenic stimuli is vital for the understanding of the regulation of T cell functions. The importance of CD28 and CTLA-4 in disease and the progression of various immune responses has been clearly shown. Further research in the future will hopefully answer some of the questions mentioned above, and help towards the developments of strategies for immunomodulation and future therapies.

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APPENDIX 1: BUFFERS AND SOLUTIONS

A. Phosphate Buffered Saline (PBS)

Five tablets were added to 500mls of mili-Q water and autoclaved to sterilise.

B. ELISA related solutions.

According to the manufacturers instructions the following buffers, diluents and solutions were made:

1. Coating buffer 0.1M carbonate, pH9.5
2. Blocking buffer 0.01M PBS pH7.3, with 4% Bovine serum albumin (BSA)
3. Wash buffer 0.01M PBS pH7.3, with 0.05% Tween 20
4. Antibody diluent 0.01M PBS pH7.3, with 0.05% Tween 20 and 1%BSA
5. Stop solution 1M H_2SO_4

C. Solutions for nuclear extractions.

The table bellow represents the synthesis of the buffers (Buffer A and C) used, the amount of each chemical used for 25mls of each buffer and the final concentration of each chemical compound in brackets

Solution.	Buffer A	Buffer C
Heppes-1M	250µl (10mM)	500µl (20mM)
$MgCl_2$-1M	37.5µl (1.5mM)	37.5µl (1.5mM)
KCl-1M	250µl (10mM)	
NaCl-5M		2,100µl (420mM)
EDTA-0.2M		25µl (0.2mM)
Glycerol		6,250ml (25%)
Mili-Q water	24,412ml	16,087ml

Both buffers were calibrated to pH 7.8 with sodium hydroxide (NaOH) and then filter sterilised. Before use the 25mls of buffers was supplemented with 25µl of 1M DTT, 25µl of 1mg/ml leupeptin and 5µl of 5mg/ml pepstatin

D. Radiolabelling oligonucleotides

For a 25µl reaction:

2.5µl oligonucleotide (1.75pmoles/ml of promega NF-kB and AP1)
2.5µl of T4 kinase 10X buffer (preferably Promega)
2.5µl γATP (0.037MBq/µl or 10µCi/µl)
1.5µl T4 kinase (Promega)
16 µl (up to 25) MQ water

For the SantaCruz oligonucleotide NFAT (20ng/µl or 1.3pmoles/µl), 3.5µl were used and 15µl water.

E. Solutions for EMSAs.

Binding reactions for EMSAs contained 12µl of the nuclear extract in buffer C (see above) together with 6µl of the binding mix and 2µl of the radio labelled DNA. The binding mix was made for all reactions and 6µl were aliquoted for each.

Binding mix

Poly dI-dC, Poly dI-dC (5µg/µl)	0.2µl (i.e. 1µg)
Glycerol	1.5µl (i.e 25% in binding mix, 7.5% in final binding reaction)
KCl (760mM) / $MgCl_2$ (12mM) the	1µl (i.e. 228mM and 3.6mM respectively in binding mix and 38mM and 0.6mM final concentrations)
Mili-Q water	3.3µl.

The binding mixtures were loaded on a large gel.

Gel composition

5mls of 30% acrylamide

0.3mls of 10% APS

0.025mls of TEMED

1.5mls of 10XTBE and

23.175mls mili-Q water.

The gel was run in 0.5X TBE buffer (0.05M Tris-Borate and 0.001M EDTA) which was diluted form a 10X stock solution.

10XTBE

108g Tris base

55g boric acid

20ml 0.5M EDTA

C. Solutions for RNA extraction.

For RNA extraction, solution D was prepared with 100g guanidinium thiocyanate resuspended in 117ml DEPC water, 7ml of 0.75M sodium citrate pH7 and 10.56ml of 10% sarcosyl. The mixture was dissolved at 65°C and stored at 4°C. Prior to use 72µl of mercaptethanol was added to 10ml of the stock solution.

APPENDIX 2: TISSUE CULTURE MEDIA

A. Dulbecco's Minimal Essential Medium (DMEM)

Mili-Q water	400mls
DMEM (10X)	55mls
FCS	50mls
Penicillin (10,000mg/ml)/	
Streptomycin (10,000IU/ml)	5mls (approximately 100µg/ml and U/ml final respectively)
Sodium bicarbonate (7.5%)	28mls (approximately 0.4% final)
Sodium pyruvate (100mM)	5mls (approximately 1mM final)
Nucleosides (100X)	5mls

Nucleosides (100X)	Thymidine (Sigma)	0.34mg/ml
	Guanosine (Sigma)	0.7mg/ml
	Adenosine (Sigma)	0.7mg/ml
	Cytidine (Sigma)	0.7mg/ml

The nucleosides were made separately at 25mls each and at quadruple the concentrations stated above. All were then warmed at 37°C in order to dissolve and were quickly filter sterilised before mixing.

B. RPMI-1640

For culturing cells 1X RPMI solution was used:

RPMI-1640	500mls
FCS	50mls
Penicillin (10,000mg/ml)/	
Streptomycin (10,000IU/ml)	5mls (100µg/ml and IU/ml final respectively)
Glutamine (200mM)	5mls (approximately 2mM final)

For washing cells with medium RPMI made from a 10X stock was used:

Mili-Q water	400mls
10X RPMI	55mls
FCS	50mls
Penicillin (10,000mg/ml)/	
Streptomycin (10,000IU/ml)	5mls (approximately 100µg/ml and U/ml final respectively)
Glutamine (200mM)	5mls (2mM final)
Sodium pyruvate (100mM)	15mls (approximately 3mM final)
Sodium hydroxide (10M)	750µl (approximately 15mM)